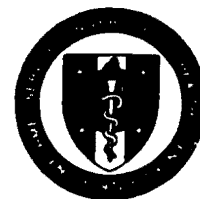# UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
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APPROVAL SHEET

Title of Dissertation: "Remote sensing and geographic information systems as decision support tools for malaria control in the Republic of Korea"

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Doctor of Public Health
30 May 2001

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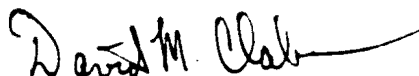
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ABSTRACT

Remote Sensing and Geographic Information Systems as Decision Support Tools for Malaria Control in the Republic of Korea

David Merrell Claborn, LCDR MSC USN

Dissertation directed by: Richard G. Andre
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Cost-comparisons are a necessary component of decision-making in the control of vector-borne disease. Remote sensing and geographical information systems (GIS) were used to estimate the size of vector larval habitats to allow a cost comparison of chemoprophylaxis and larviciding, two malaria control methods currently being considered for use in the Republic of Korea (ROK).

Two U.S. Army camps (Cp Casey and Cp Greaves) were selected as research sites. The cost of chemoprophylaxis was estimated for each population assuming a 19-week treatment regimen consisting weekly chloroquine chemoprophylaxis with terminal primaquine treatment, a single pre-treatment G-6-PD deficiency test, and a 50% turnover in personnel during the malaria transmission season. Annual cost of chemoprophylaxis was $37.53/person.

Larval habitats were sampled from June through September, 2000. Anopheline larvae were reared to the

adult stage and identified using adult and pupal
morphological characteristics. Gene sequencing and random
amplification of polymorphic DNA (RAPD) analyses were
performed to confirm the identification of the mosquitoes.
Both molecular work and pupal morphology indicated that
most of the anophelines collected during the study were
Anopheles sinensis Wiedemann and that the abundance of
other anopheline species is generally overestimated by
adult keys.

The size of vector larval habitats within the 1-km
flight range of *An. sinensis* around the two U.S. Army camps
was determined using satellite-acquired images, and the
cost of treating those areas with an insect-growth
regulator was estimated. At Cp Greaves, the cost of the
requisite three larvicidal applications exceeded the cost
of chemoprophylaxis by a factor of four, but at Cp Casey,
chemoprophylaxis was about 21 times as expensive as
larviciding due to the larger number of at-risk personnel
and the smaller size of the vector habitats.

This study demonstrates the usefulness of remote
sensing and GIS as decision support tools for estimating
costs of vector control methods used in the control of
vector-borne disease. Such information can then serve as

one component of the decision-making process for the design

and implementation of disease prevention strategies.

Remote Sensing and Geographic Information Systems as

Decision Support Tools for Malaria Control in the Republic

of Korea.

By

David Merrell Claborn
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Dissertation submitted to the Faculty of the Department of

Preventive Medicine and Biometrics of the Uniformed

Services University of the Health Sciences in partial

fulfillment of the requirements for the degree of Doctor of

Public Health.

May, 2001

ACKNOWLEDGEMENTS

opposite can occur. None see this more than the family members. To my wife and daughter I express my sincerest gratitude for their tolerance and sacrifices during the completion of this dissertation.

Finally, I am greatly indebted to the U.S. Navy for allowing me to return to school to complete my doctoral program. I would never have achieved this long sought after goal without the opportunities provided by this wonderful organization.

TABLE OF CONTENTS

CHAPTER 6

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

General Introduction

GENERAL INTRODUCTION

The Presidential Decision Directive NSTC-7

(National Science and Technology Council) of 1996 formally

expanded the role of the Department of Defense (DoD) in the

support of global surveillance, research and response to

emerging infectious disease threats. As a result, the DoD

Global Emerging Infections Surveillance and Response System

(DoD-GEIS) developed a five-year strategic plan with four

goals addressing surveillance, systems development,

response and capacity building (Walter Reed Army Institute

of Research, 1998). Goal 1 was to "detect and monitor

emerging pathogens, the diseases they cause, and the

factors influencing their emergence to protect military

readiness, the health of DoD beneficiary populations, and

other national interests". Goals 2 and 3 dealt with

enhancing infrastructure for prevention and control

strategies. Goal 4 logically followed with efforts to

"leverage DoD and international public health

infrastructures through training, networking, and other

forms of assistance to support surveillance, assessment,

response, and prevention of emerging diseases." One

emerging disease threat specifically mentioned for DoD

attention was the re-occurrence of malaria (*Plasmodium vivax*) along the Korean Demilitarized Zone (DMZ).

After an absence of more than ten years, malaria re-emerged in the Republic of Korea (ROK) in 1993 (Feighner et al. 1998). The number of detected cases grew from one in 1993 to 3,719 in 1999. Of all cases diagnosed in the ROK through 1999, 83 (2.23%) were among American military personnel or Korean augmentees to the U.S. Army. As of December 2000, sixteen confirmed malaria cases in American personnel stationed in the ROK had occurred for that year alone (Preventive Services Directorate, 18th Medical Command, personal communication). The malaria cases have been concentrated just south of the DMZ that separates North and South Korea and where large populations of American and Korean military personnel are stationed.

Reasons for the re-emergence of malaria in the ROK are unknown. Ree (2000) speculated that most malaria cases resulted from the bite of mosquitoes that dispersed across the DMZ from the PDRK. This scenario seems doubtful given the limited flight range of the mosquito and the number of cases that have occurred. Few anti-malaria efforts have been in effect in the ROK since the the late 1970's so a re-emergence of the disease might not be surprising.

However, the re-emergence of malaria reflects a trend over a much larger area than just the Korean Peninsula. Several areas in eastern Asia, especially parts of China, are also experiencing the re-emergence of malaria. In Henan province just south of Beijing, indoor spraying with DDT to reduce populations of the vector ceased after malaria cases dropped from a total of 10.2 million in 1970 to 318 in 1992 (Sleigh et al. 1998). Since 1994, when spraying ceased, *Plasmodium vivax* has resurged to infect nearly 13% of the population in some communities. Even some cases of *P. falciparum* have been detected (Zizhao et al. 1999). As with many other Chinese provinces, the primary malaria vector in Henan province is *Anopheles anthropophagous* Xu and Feng, a strongly anthropophagic and endophilic[1] species. The vectorial capacity of *An. anthropophagous* has been estimated to be 20 times that of the secondary vector, *An. sinensis*. The latter is much more zoophagic and exophilic, and perhaps due to these differences between the primary and secondary vectors, malaria in China is often well controlled with a combination of indoor sprays and insecticide-impregnated bed nets (Cheng et al. 1995, Sleigh et al. 1998). In fact, Depang et al. (1996) contended that vector control in the form of residual

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[1] Vector efficiency is a function of the mosquito's tendency to feed on humans and enter human houses.

DDT sprays and treated bed nets was primarily responsible for significant declines in malaria rates throughout Xinyang province in far western China. The situation in the ROK is much different because the primary vector is *An. sinensis*. Unlike many other members of the '*hyrcanus*' complex that commonly are found indoor, the exophilic nature of *An. sinensis* renders it less susceptible to indoor spraying. This exophilic nature, as well as the tendency of this species to feed on large non-human mammals, reduces its capacity to act as a vector in the transmission of malaria. In addition, *An. sinensis* is refractory to infection with some species of *Plasmodium*, specifically *falciparum* and *yoelli* (Rongariyam et al. 1998), although it is weakly susceptible to infection with *P. vivax*.

The strain of *P. vivax* transmitted in the ROK is well adapted to a temperate climate and demonstrates both short and long incubation periods. Long incubation periods increase the risk of re-introducing malaria to the continental United States, making malaria control in the ROK even more important (Walter Reed Army Institute of Research 1998). This increased risk is from U.S. soldiers contracting malaria with parasites that undergo long incubation, then returning to the continental U.S. where

they become ill and where competent vectors exist. Such importations were common after the Korean War (Ree 2000).

The DoD-GEIS goals and the specific circumstances of malaria resurgence in the ROK initiated an interest in designing and implementing cost-effective disease control measures for U.S. military personnel on the Korean peninsula. Several malaria control methods have been used throughout the world, including water management, adulticidal insecticides, repellents, mosquito nets and more (Chin 2000), but the two methods under consideration for expanded use in the ROK are chemoprophylaxis and larviciding.

The Uniformed Services University of Health Sciences (USUHS) and the National Aeronautics and Space Administration (NASA) have collaborated on investigations of remote sensing and GIS techniques to monitor vectors and vector-borne disease in Mexico, Belize and, more recently, Peru (Roberts et al. 1999, Rejmankova et al. 1998, Roberts and Rodriguez 1994, Masuoka et al. 1998). These projects investigated the detection of habitats associated with vector populations or human disease with satellite-derived imagery of the locale under study. The information from these projects was useful in targeting control measures such as insecticides for vector control. Researchers at

USUHS and NASA (including Dr. Richard Andre, Dr. Donald Roberts and Mrs. Penny Masuoka) thought that the situation in the ROK was also suitable for such research, especially with regard to the use of larvicides in vector habitats near American military establishments. The clumped distribution of larvae described by Strickman et al. (1999) led those authors to suggest larviciding as a possible control measure, but the cost of implementing such a program is difficult to determine. Remote sensing provides one method of obtaining a cost estimate.

The Taxonomy of *Anopheles* in Korea

Of the four *Anopheles* species commonly found in the ROK, three are in the *An. hyrcanus* complex; only *An. sineroides* Yamada is not a member. *Anopheles sinensis, An. lesteri* Baisas and Hu, and *An. yatsushiroensis* Miyazaki all share the definitive morphological characteristics of the '*hyrcanus*' complex as described by Reid (1953):

> "...female with tufts of scales on the clypeus on each side, palps with pale bands (usually four) one of which is always apical, seventh sternite with a tuft of scales, fifth hind tarsus not all white (rare exceptions in *argyropus*) stem of vein five always with a well defined dark mark towards the base, male phallosome leaflets with some serrations, and of unequal length, pupal trumpet without a secondary

7

cleft, outer clypeal hairs of larva with 30-40 branches….."

The 'hyrcanus' complex occurs in an enormous geographical area, from Spain to the Pacific coast of Russia, all the way to the Oriental region in the south. Palearctic and Oriental representatives of the complex are separated by geographical features running from the Indian Ocean up through the Afghanistan peaks and just east of the Himalayas. The Oriental members of the complex, which are the focus of this dissertation, include the majority of the species, including *An. nigerrimus* Giles, *An. pursati* Laveran, *An. indiensis* Theobald, *An. pseudosinensis* Baisas, *An. argyropus* Swellengrebel, *An. lesteri lesteri, An. lesteri paraliae* Sandosham, *An. crawfordi* Reid, *An. peditaeniatus* Leicester, *An. yatsushiroensis, An. liangshanensis* Kang, Tan, Cao, Cheng, Yang and Huang, *An. xiaokianus* Ma, and *An. sinensis.* *Anopheles anthropophagus* was recently separated from *An. lesteri* in China and is considered to be the primary malaria vector in much of China (Bo et al. 1999). A new species, *An. nimpe* Nguyen, was recently described in Vietnam (Nguyen et al. 2000).

Larvae of the 'hyrcanus' complex are found typically in swampy habitats, specifically in habitats with very slow moving or stagnant water and some emergent

vegetation. They are rarely found in jungle habitats,
although Harrison and Scanlon (1975) noted that
representatives of the complex do occur in mountainous
areas where small rice paddies exist. Different species
are often sympatric but have different preferences for
larval habitat. Members of the complex exhibit great
diversity in tolerance of salinity and temperature. One
species (lesteri) reportedly has been collected from water
with salinity as high as 82 percent that of seawater
(Harrison and Scanlon, 1975). Similarly, some members of
the complex thrive in temperatures up to 41^0 C, a
temperature that would kill many other mosquitoes. This
latter adaptation is clearly advantageous for utilization
of rice fields as larval habitats.

Adults are primarily zoophilic, with some
exceptions like An. anthropophagus that tends to be
antrhopophilic. Most members of the complex are reluctant
to enter houses (exophagic), which may limit the
effectiveness of residual interior sprays for malaria
control in cases where some members of the 'hyrcanus'
complex are the primary vectors. This conclusion appears
to be valid with regard to An. sinensis because the density
of this species was previously unaffected by residual DDT
sprays in Korea (Chow 1973). (Recent work indicates that

the primary malaria-prevention activity of DDT is due more to repellency than to toxicity (Bangs 1999, Roberts et al. 2000, Grieco et al. 2000). Nevertheless, the efficacy of interior hut sprays in prevention of malaria where the primary vector is exophagic is defined poorly at present.)

The role of the 'hyrcanus' group in malaria transmission is still controversial. The presumed primary vector in the ROK, *An. sinensis*, has long been regarded as a vector of malaria in China as well. At times, it is the only vector present or detected (Reid 1953). Chow (1950) stated that it is the principal vector in central China but becomes secondary to *An. minimus* Theobald in southern China. A similar situation occurred in Taiwan where *An. minimus* was the primary malaria vector in the foothills. In fact, throughout much of southeast Asia, the 'hyrcanus' complex contributes secondary vectors while other species including *An. minimus, An. sundiacus* (Rodenwaldt), and *An. leucophyrus* Doenitz appear to serve as the more important malaria vectors (Reid 1953).

Assessment of the vectorial capacity of the 'hyrcanus' complex has changed somewhat since the recent taxonomic separation of *An. anthropophagus* and *An. lesteri*. In Sichuan, China, where *An. anthropophagus* is sympatric with *An. minimus*, the former is considered to be a vector

of very high potential (Liu et al. 1986). This conclusion

is based, in part, on the affinity of *An. anthropophagus*

for human habitations, especially when compared to *An.*

sinensis. Night time counts revealed that nearly 70% of

the anopheline mosquito population in human habitations was

An. anthropophagus; whereas, less than 30% was *An.*

sinensis. In contrast, *An. sinensis* comprised more than

90% of the *Anopheles* in cowsheds and *An. anthropophagus*

less than 5%. The human blood index, which is a measure of

the proportion of mosquitoes that have fed on humans, also

implicates *An. anthropophagus*. The index for this species

was 0.291 to 0.633 for *An. minimus* as compared to 0.0597 to

0.119 for *An. sinensis* (Wang et al. 1987). Natural

positive sporozoite rates for *An. anthropophagus* were 0.34%

to 0.54% in Guandong province (Bo et al. 1999) and 1.9% in

Guangxi (Wang et al. 1987). These last two studies did not

quantify sporozoite rates for *An. sinensis*, but the

secondary status of this species in the study areas was

assumed. However, in Jining, China, the incidence of

malaria and the serum antibody levels of the residents were

lower in areas with low densities of *An. sinensis* (Yi and

Wang 1999).

Historical difficulties with the identification of

mosquitoes within the '*hyrcanus*' complex place the findings

of many studies in question. In particular, studies that include both *An. sinensis* and *An. lesteri* are problematic due to difficulties with separating these two species. Three characteristics (two adult and one pupal) used by Otsuru and Ohmori (1960) to separate these two species were evaluated using specimens from Hong Kong and were found to be useless by Harrison (1973). Yet, one of these characteristics, the presence or absence of a pale fringe spot at the termination of wing vein Cu_2, is still an important characteristic in taxonomic keys by Tanaka et al. (1979), Connor and Soeponto (1979), Toma and Miyagi (1986), and Lee (1998). Each of these keys is written for the identification of mosquitoes from a limited geographic area (Japan/Korea, Indonesia, the Ryukyu Archipelago, and the Republic of Korea, respectively). It may be that this characteristic is reliable in some geographic areas but not in others, and an application of characteristics written for one area to identify specimens from another may result in misclassifications.

With the advent of molecular studies, the relationships of some of the '*hyrcanus*' complex have been better defined. Baimei et al. (1993) compared the metaphase karyotypes of six '*hyrcanus*' species, noting that both *An. sinensis* and *An. crawfordi* have two forms of the

karyotypes. This finding is consistent with field observations by Reid (1953), who suggested that there are at least two forms of *An. sinensis*, which he termed "Palearctic" and "Oriental", and that they differ in their ability to serve as malaria vectors. Li et al. (1991) used restriction fragment length differences of repetitive DNA to produce unique fragments for five '*hyrcanus*' species including *An. sinensis*, *An. anthropophagus*, *An. liangshanensis*, *An. crawfordi* and *An. xiaokuanus*. This technique uses restriction enzymes to cut DNA into precise lengths that can be separated on a gel. A radioactive probe can then be used to confirm the presence of a length of a specific size. These lengths can be used to separate these sibling species.

Two other powerful molecular techniques that have been used to investigate the relatedness of sibling species and related groups are randomly amplified polymorphic DNA (RAPD) and the polymerase chain reaction (PCR), the former actually being a special type of the latter. The purpose of a PCR reaction is to make numerous copies of a gene for subsequent sequencing or other molecular procedures. There are three primary steps in a PCR (denaturation, annealing and extension) that are repeated for 30 or 40 cycles. During the denaturation, the double strand of DNA is

13

separated into single stands. In the annealing step, short primers are ionically bonded with the single strands, with the best fitting primer-strand bonds lasting longer. A polymerase attaches to the most stable double strands, copying the template. During the extension steps, bases that are complementary to the template are coupled to the primer and the template. These cycles are repeated in an automated cycler that has the ability to change temperatures in the PCR mixtures rapidly and accurately. After 30-40 cycles, there is an exponential increase in the copies of the original DNA that are then available for further investigations (**http://www.highveld.com/pcr.html**). In RAPD analysis, parts of the DNA are amplified using single oligonucleotide decamer primers of arbitrary sequence. The amplified fragments are separated using agarose gel electrophoresis and visualized by ethidium bromide staining. This technique regularly produces bands in an agarose gel that are fixed and unique to a particular species; members of a species can be identified because they share unique multiple bands.

Cornel et al. (1996) used PCR techniques to develop a diagnostic assay to distinguish cryptic sibling species of the *An. quadrimaculatus* Say complex. A similar technique was used by Porter and Collins (1991) to

distinguish between the morphologically similar *An. freeborni* (Aitken) and *An. hermsi* Barr. Hettiaratchi et al. (2000) identified a primer that amplified only *Culex quinquefasciatus* Say DNA, making an ideal diagnostic tool for identification of this species. A restriction endonuclease was used by Bortel et al. (2000) to digest DNA from members of the *An. minimus* complex. Diagnostic banding patterns in agarose gels were obtained that separated all six of the species tested. The RAPD technique was used to investigate the *Ochlarotatus japonicus* (Theobald) complex in the United States (Fonseca et al. (in review)), the *An. albitarsis* Lynch-Arribalzaga in Paraguay, Argentina, and Brazil (Wilkerson et al. 1995), and *Aedes aegypti* of Puerto Rico (Apostol et al. 1994). Although these techniques have not yet been applied to studying the difficult taxonomy of the '*hyrcanus*' complex, they do have great potential for solving some of the questions regarding the identification of specimens from the complex. As part of the current study, gene sequencing techniques and RAPD analysis will be used to supplement a morphological comparison of female adult anophelines and pupal exuviae from immatures collected in mosquito habitats of the ROK. The purpose of this part of the study is to determine the relative occurrence of two species: *An.*

sinensis and *An. lesteri*. This information is essential to the study because the vector status of *An. lesteri* in the ROK is unknown. As these two species are putatively the most numerous in the area, their abundance in larval habitats is an essential part of determining productivity and size of vector habitats in the ROK.

Chemoprophylaxis as an Option for Control of Malaria among U.S. Army personnel in the Republic of Korea

Malaria chemoprophylaxis is the use of medication to prevent or suppress *Plasmodium* infection in the erythrocytes of the human host. With *P. vivax* and *P. ovale*, chemoprophylaxis must also eliminate the hypnozoite in the human liver. The widespread use of antimalarial drugs for chemoprophylaxis, accompanied by poor compliance and underdosing, has provided the selection pressure for emergence of resistance to many regularly prescribed drugs (Brown 1993). However, the need for American military personnel to use chemoprophylaxis in many malarious areas remains evident. Military personnel, like other international travelers, often lack the degree of immunity acquired by residents of malarious areas and are at increased risk of malaria morbidity and mortality from this disease.

The issues of drug resistance and the possibility
of drug side effects require a variety of options in
prescribing chemoprophylaxis to military personnel. The
U.S. Navy describes three basic regimens (Navy
Environmental Health Center 1998):

Regimen A: In areas without chloroquine-resistant
P. falciparum, use a   weekly dose of chloroquine
(500-mg salt). Begin the regimen two weeks before
entering the area and continue for four weeks
after leaving.

Regimen B: In areas with chloroquine-resistant *P.
falciparum*, use mefloquine (250 mg/week). Begin
regimen two weeks before entering the area and
continue for four weeks after leaving. This
regimen is not for those with a history of
seizures, psychiatric disorders, or cardiac
conduction abnormalities and is contraindicated
for pilots and others on flight crew status.

Regimen C: Doxycycline is also for use in areas
with chloroquine resistant malaria. Use a daily
dose of 100 mg/day, starting 1 to 2 days prior to
entering the endemic area and continuing daily
doses for four weeks afterwards.

All of these drugs attack the erythrocytic stage of the parasite, so terminal prophylaxis may be required if the patient is at risk of infection with *P. vivax* or *P. ovale*. Primaquine was the only drug available for such use in the United States until July 2000 when malarone was approved by the American Food and Drug Administration **(http://pharmacology.about. com/health/pharmacology/library/0daily/00news/blnews00 0714_malarone.htm)**. This latter drug is a combination of atovaquone and proguanil and has been shown to be effective against the early liver stage of the parasite. However, current Army chemoprophylaxis recommendations in the ROK include only the use of primaquine for terminal use.

Although generally safe and effective, chemoprophylaxis is not without its drawbacks. With the advent of chloroquine-resistant *P. falciparum*, no chemoprophylactic regimen is completely effective (Bruce-Chwatt 1982, Rogerson et al. 1994). Even if prescribed drugs are taken regularly and appropriately, complete protection from malaria cannot be expected. This situation places the public health official in the dilemma of placing a large number of people on chemoprophylaxis while knowing that this act may increase the selection pressure on the existing mutants with resistant qualities. Reliance on

chemoprophylaxis alone has proven to be increasingly difficult and most modern programs now rely on a variety of control measures rather than just the administration of drugs (Bia, 1992).

Another issue that detracts from the effectiveness of chemoprophylaxis is that of non-compliance. One study of non-military travelers to Africa indicated that only 52% claimed to have taken all of their chemoprophylactic medications with no missed doses (Behrens et al. 1998). Moreover, the effect of incomplete chemoprophylaxis was roughly equivalent to no chemoprophylaxis at all. Similar levels of non-compliance have been noted for travelers from Canada (31%) (dos Santos et al. 1999) and the Netherlands (60%) (Cobelens and Leentraar-Kuijpers, 1997). In U.S. Marines deployed to Somalia in the early 1990's, self-reported chemoprophylaxis compliance was only 56% (Newton et al. 1994). Given that 112 cases of malaria occurred in these Marines, it is obvious that non-compliance "contributed to what was the largest outbreak of imported malaria in U.S. military personnel since the Vietnam conflict."

Reasons for non-compliance among travelers to India included poor pre-travel medical advice, side effects of the drugs, active decisions against adequate advice, confusion about alternate regimens and perceived uselessness of the drugs (Chatterjee

1999). The applicability of these reasons to military personnel is unknown, but similar non-compliance with the use of personal protective measures suggests a widespread lack of concern about contracting malaria. Over 80% of a sample of Singaporean Armed Forces felt that military-issued repellents (the same as those issued by the U.S. military) were inadequately protective against mosquito bites (Fai 1996). A surprising 20.7% stated they would not even bring the repellent with them during field exercises, and 55.5% stated that they used the repellent rarely or not at all. A majority (70.4%) indicated they used and preferred commercial repellents for a variety of reasons, including efficacy, convenience, odor, persistence, and less damage to plastics. This latter observation is consistent with my personal observations of deployed U.S. Naval personnel in Australia and Saudi Arabia, where some U.S. Marines and Sailors preferred to buy expensive local repellents or flea-repellent dog collars rather than use military-issued repellents.

Another potentially serious problem with the use of primaquine for terminal prophylaxis of the pre-erythrocytic stage of the parasite is severe hemolytic anemia in persons who are deficient in glucose-6-phosphate dehydrogenase (G-6-PD). Primaquine is the only drug currently used by the U.S. Army that

destroys the hypnozoites in the liver, although malarone is also effective. It is a very strong oxidizer. Oxidation results in the denaturation of hemoglobin, resulting in hemoglobinuria, kidney damage and anemia. Erythrocytes are protected from oxidation by the hexose monophosphate shunt, but in the G-6-PD deficient individual, protection is insufficient.

There is no uniform policy in the military regarding screening for G-6-PD deficiency. U.S. Navy policy requires that all personnel be tested upon entry into the service. Although primaquine can be used safely in the G-6-PD deficient individual under close supervision from a physician, current Navy policy prohibits such use. Army personnel are not routinely tested for G-6-PD deficiency, though Air Force personnel are tested upon entry.

Although *P. vivax* exhibits significant resistance to the standard drug, chloroquine sulfate, the Korean strain is still susceptible and this drug continues to be used as part of the U.S. Army's malaria chemoprophylaxis program (Preventive Services Directorate, 18th MEDCOM, unpublished data). During 1999, U.S. soldiers and augmentees training in high-risk areas (i.e. areas exhibiting recent malaria transmission) of the ROK were placed on chloroquine prior to exercises until the end of the malaria season in October. During calendar-year 2000, only U.S.

soldiers residing north of the Imjim River received
chemoprophylaxis. Terminal prophylaxis consisted of
primaquine during both years. Soldiers who were at-
risk for only a few days relied on personal protective
measures (repellents, protective uniform, etc.) and
did not receive chemoprophylaxis.

The efficacy of chloroquine chemoprophylaxis
in the ROK has not been qualified and the decision to
use the drug was based in part on the continuing
efficacy of the drug in treatment of active malaria
infections. Table 1 describes several studies on the
use of chloroquine and primaquine both as treatment
and chemoprophylaxis in various countries.

Table 1. Recent studies on chloroquine and primaquine for treatment and prevention of malaria

Location	Population	Parasite	Findings	Citation
India	Children	P. falciparum	100% "good response" to chloroquine treatment	Nandi and Sharma, 2000
India	Open	P. falciparum	>50% treatment failure to chloroquine treatment	Kshirsagar et al. 2000
India	Open	P. vivax	26.7% relapse rate with chloroquine treatment	Gogtay et al. 1999
Nigeria	Children	P. falciparum	92.7-98.2% cure rate with chloroquine	Sowunmi et al. 2000
Nigeria	Open	P. falciparum	chloroquine less effective than quighaosu	Ezedinachi 1996
Nigeria	Children	P. falciparum	60% failure rate with chloroquine treatment	Falade et al. 1997

Table 1. (Con't)

Location	Population	Parasite	Findings	Citation
Indonesia	Trans-migrants	*P. vivax* & *P. falciparum*	Malaria risk 3.96-10.56 times higher in trans-migrants receiving chloroquine rather than primaquine chemopropholaxis	Baird, et al. 1995
Kenya	Pregnant women	*P. falciparum*	46% chloroquine resistance	Rukaria-Kaumbutho 1996
Kenya	Children	*P. falciparum*	71% chloroquine resistance	Anabwani et al. 1996
Brazil	Adult	*P. falciparum*	100% chloroquine resistance	Segurado 1997
Myanmar	Children	*P. falciparum*	Chloroquine failure rate five times that of mefloquine	Ejov et al. 1999

Table 1. (Con't)

Location	Population	Parasite	Findings	Citation
Germany	Travelers	P. vivax	12.5% relapse rate with primaquine treatment	Jelinek 1995
Thailand	Adult males	*P. vivax*	8-17% relapse or recrudescence with chloroquine treatment	Pukrittaykamee 1994
Zimbabwe	Children	*P. falciparum*	52% chloroquine failure rate	Mharakurwa et al. 1998
Cameroon	Adults	*P. falciparum*	56% chloroquine failure rate	Ringwald et al. 1996
Columbia	Children	*P. falciparum*	44% chloroquine failure rate	Osorio et al. 1997
Columbia	Soldiers	*P. falciparum*	88% protective efficacy of chloroquine/primaquine chemoprophylaxis; not different from primaquine alone	Soto et al. 1999

Use of Larvicides as an Option for Control of Malaria in the Republic of Korea

Some U.S. Army public health officials in the ROK speculated that an environmental control method might provide better malaria control in those areas with transient military populations. Strickman et al. (2000) suggested that larviciding in mosquito habitats around American installations might be a viable control method if such efforts were coordinated with Korean civilian authorities. One advantage of larviciding would be that protection could be conferred on all U.S. and ROK soldiers at-risk, regardless of the length of exposure. However, a considerable amount of data on the bionomics of vector larvae in the malaria endemic areas would have to be gathered to determine the feasibility and cost of such a larviciding program. To determine the cost of a larviciding program, the size of the area to be treated should be known, but before the size and location of larval habitats can be determined, three questions about the local vectors of malaria must be answered

(1)   Which mosquitoes are proven vectors?

(2)   Which species of vectors occur in the larval habitats?

(3)   Where do the vector larvae occur?

In a review of Korea's arthropods of public health importance, Chow (1973) mentioned only two potential malaria vectors: *An. sinensis* (Wiedemann) and *An. yatsushiroensis* Miyazaki. Of these two species, Chow considered the former to be the most important vector.   This conclusion has generally been considered correct by subsequent authors, but the unclear taxonomy of *An. sinensis* and others in the '*hyrcanus*' group has caused significant confusion.   In particular the difficulty distinguishing *An. sinensis* from *An. lesteri* is important because the vector potential of *An. lesteri* is unknown.   Tanaka et al. (1979) considered *An. lesteri* to be the probable primary vector of malaria in Japan, rather than *An. sinensis*, but *An. lesteri* has not been investigated extensively on the Korean peninsula.

In a survey of malaria vectors in Kyonbuk, Korea (Joo and Kang, 1992), *An. sinensis* was the only species considered, even though it is generally described as strongly zoophilic.   Ree et al. (1973) reported only three *Anopheles* species caught in light traps (*An. sinensis, An. sineroides* Yamada, and *An. yatsushiroensis*) with *An. sinensis* comprising 95% of the anophelines and 18% of the

27

total mosquito population. Strickman et al. (1999) caught the same three species using light traps in the northern region of South Korea, but also caught one specimen identified as *An. lesteri*. In that study, larval surveillance also indicated densities of from 0.02 - 3.1 larvae/dip in rice fields surrounding a military training where local transmission had probably occurred. A later study by some of the same authors (Strickman et al., [in review]) caught many more adult *An. lesteri* and *An. yatsushiroensis*, none of which were positive for *P. vivax* circumsporozoite protein. In fact, only two out of 2,376 *An. sinensis* tested for malaria infection using circumsporozoite ELISA tests were positive. Therefore, *An. sinensis* is not considered to be an efficient vector, but its relative abundance and the detection of *P. vivax* only in this species implicates this mosquito as the primary vector in the ROK.

Most of the previously mentioned studies dealt with the biology of anopheline adults. Little specific information has been gathered on the biology of larval mosquitoes in the ROK, but such information is essential for any potential larviciding program in the area.

Larviciding programs for the control of malaria and malaria vectors are somewhat controversial. Successes have

occurred, including the elimination of *An. gambiae* from
Brazil using Paris Green and the combined efforts of the
Brazilian government and the Rockefeller Foundation in the
1930's (Burgess 1981). Other early successful programs
include the British efforts in India and the American
program in the Panama Canal Zone (Crawford and Chalam,
1926). The following quote from W.C. Gorgas (1903) is
indicative of early attitudes toward larviciding for malaria
control:

> I would require, that on receiving a report that
> mosquitoes were bad in a particular house, that
> the inspector locate the cause on the premises.
> In many instances, it occurred that the inspectors
> had to be sent back several times, with orders to
> look more carefully over the premises. I do not
> think there was a single instance, in which the
> mosquitoes were reported as being unusually bad,
> in which the cause was not located on the premises
> immediately concerned. For the year 1900, the
> year preceding the beginning of our mosquito work
> in Havana, we had 325 deaths from malaria. In
> 1901, the first year of our mosquito work, we
> reduced this by half, and had 151 deaths from
> malaria. The second year of mosquito work, we
> reduced the results of the preceding year again by
> half, and had 77 deaths, and up to the 1st of
> September, 1903, I see from the Havana health
> reports that they have had but 39 deaths from
> malaria. This is a very fair measure of the
> amount of mosquito work done and the results
> obtained from this class of work, because, from
> the nature of the disease, the malarial patient
> could not be isolated and followed up, as was the
> yellow fever case, and therefore, no special work
> could be done toward destroying the malarial
> infected mosquito. The decrease in the number of
> malarial cases is entirely due to the decrease in
> the number of mosquitoes at large. In many parts

of Havana, when I left there in October, 1902,
mosquitoes had entirely disappeared. The
inspectors of the various districts made daily
reports of the condition of the houses inspected
by them, as to whether or not there was mosquito
larvae on the premises…The consolidated report of
January 1901, just before our mosquito work
commenced, showed 26,000 water deposits containing
mosquito larvae within the city limits. The same
consolidated report for the following January
showed less than 300 for the same area.

With the advent of DDT and other insecticides,

however, the use of larvicides like Paris Green, an

inorganic arsenical, became less important. Weidhaus and

McDuffie (1968) stated that "larvicides should only be used

when the sources of larval breeding cannot be eliminated or

as a temporary measure until source reduction can be

undertaken…larvicides should be used only where and when

they are needed and where other methods cannot be employed

or will not give effective control."

Many of the reservations about the use of

larvicides concerned undesired effects on non-target

organisms, such as fish and invertebrates. In addition, the

early effectiveness of interior DDT sprays against

endophilic vectors discouraged the use of other, more

expensive techniques such as larviciding. With the

development of mosquito resistance to DDT, accompanied by

concerns about its environmental effects, interest in

alternate control methods re-emerged. Control agents with high specificity for target organisms and low impact on the environment were developed in the 1970's and 1980's. Biologic agents such as *Bacillus thuringiensis israeliensis* (Bti), *Bacillus sphaericus*, and methoprene (an insect growth regulator) increased interest in the use of larvicides for control of mosquito larvae. Several were even tested for larval control in the rice field environment. These tests are particularly important because the rice fields serve as an important habitat for the primary malaria vector in the ROK, *An. sinensis*.

The strong association of *An. sinensis* with rice cultivation has been noted many times (Strickman et al. 1999, Tanaka et al. 1979, Harrison and Scanlon 1975). Although not limited to this environment, *An. sinensis* develops large populations in rice paddies, and reductions in rice culture have been linked to reductions in this species' populations (McDonald and Savage 1972, Claborn 1995). In addition, the finding that larvae have a clustered distribution in rice fields led Strickman et al. (1999) to suggest that larvicides could be targeted in those fields that produce the most mosquitoes. However, Joo and Kang (1992) demonstrated strong resistance in *An. sinensis* larvae to three out of four organophosphate

insecticides that were tested. Only fenitrothion still caused significant mortality to caged specimens. Such resistance is common where mosquito larvae are incidentally exposed to agricultural compounds, and it suggests that an effective, long-term control of mosquito larvae would not be feasible with neurotoxic insecticides such as chlorpyrifos and malathion.

Fortunately, the "biological insecticides" previously mentioned are available and have proven to be effective in certain situations. Some of the first commercially available examples were of *Bacillus* formulations with specific action against a limited range of insects. Two species of *Bacillus*, *B. sphaericus* and *B. thuringiensis* variety *israeliensis* (Bti), have been tested and used with some success in a variety of larval habitats, including rice fields. In India, a substantial decline in malaria cases was attributed to the weekly application of *Bti* combined with the use of larvivorous fish for control of rice-field mosquitoes (Kumar et al. 1994). A similar study in California noted significant reduction of mosquitoes in rice fields treated with *Bti* and the mosquito fish, *Gambusia affinis* (Kramer et al. 1988); however, fields without mosquito fish experienced a rebound of the larval populations to pre-treatment levels within two

weeks.   In contrast, in Arkansas *Bti* achieved 100% control of *Psorophora columbiae* (Dyar and Knab) in small rice plots without the use of mosquito-eating fish (Meisch et al. 1990).

Another bacterium, *B. sphaericus* also has been tested in rice fields and other habitats with mixed results (Sundararaj and Reuben 1991, Karch et al. 1992).   Unlike *Bti*, which cannot multiply in the environment, some strains of *B. sphaericus* persist and recycle in aquatic habitats. Cadavers of infected larvae contribute to the persistence of *B. sphaericus* because they contain all of the nutrients necessary for both vegetative multiplication and toxin synthesis by the bacterium (Becker et al. 1995).   This persistence has two potential benefits: (1) the need for fewer applications per growing season and, (2) the potential for evolution to counteract resistance in the insect.   Another advantage to the use of *B. sphaericus* is its efficacy in water with high organic content.

Numerous efficacy tests have been performed on *B. sphaericus* in a variety of habitats.   A bacterial suspension achieved up to a 79% reduction of *An. fluviatilus* James and *An. culicifacies* Giles larvae in stream pools in India (Mariappan et al. 1999).   The product was not recommended for *Anopheles* control due to

inconsistent results, though good control was obtained for *Culex quinquefasciatus* Say. Weekly re-applications of *B. sphaericus* were required to maintain suitable control of *An. culicifacies* in rural parts of Uttar Pradesh, India (Sharma et al. 1998). Weekly applications also were required to control *An. stephensi* Liston in masonry tanks, sump tanks, and other urban habitats in Goa, India, but a significant reduction in the number of malaria cases was associated with these treatments (Kumar et al. 1994).

An insect growth regulator, methoprene, also has been tested in rice plots in Arkansas and has achieved long-term control (98.2% reduction in mosquito larval population for at least two months) with a slow-release briquette formulation especially applicable for use in rice fields (Weathersbee and Meisch 1991). Methoprene also was highly effective in controlling both *Culex* and *Anopheles* larvae in the ponds and ditches of Assam, India (Baruah and Das 1996). When applied for control of *Ochlerotatus triseriatus*[2] (Say) in waste tires in Illinois, methoprene and *B. sphaericus* had similar high levels of control for a mean of one month (Siegel and Novak 1999). *Although B. sphaericus* was cheaper, the methoprene formulation (briquette) was easier to apply.

An additional benefit of the biological insecticides and insect growth regulators is their restricted toxicity. When *Bti* and methoprene were applied to mangrove swamps in Florida, there was no detectable mortality of amphipods or non-target insects, even though good control of *Aedes taeniorhynchus* was observed (Lawler et al. 1999).

Any larviciding program on the scale required for malaria control in the ROK would be costly. Even limiting the treated areas to a one or two kilometer radius around the American bases (the estimated flight range of *An. sinensis*) could still require treatment of several hectares. A long-term control program using larvicides might not be economically feasible, but even a very high expense might not prevent the contingent use of larvicides in the event of epidemic disease or, perhaps, a wartime scenario. The cost of larval control programs can be minimized with the precise targeting of larvicides in habitats that serve as breeding sites for the mosquitoes. Such targeting not only reduces the cost of application, but also minimizes and potential undesired environmental effects, such as unintended bird mortality. In the past,

such targeting has been achieved through labor-intensive

"field scouting" in which a worker samples the water in the

larval breeding site and quantifies the number of larvae in

the sample. Identification of the mosquito was usually

performed later, either through microscopic examination of

the immature phase or through identification of the adult

after being reared to maturity in the laboratory. Field

sampling is labor-intensive and expensive, especially when

large areas are involved. It may not be feasible where

labor is limited or expensive, or where the technical

expertise is lacking. An alternative to field scouting and

sampling is the use of remote sensing technology.

Remote Sensing and Geographic Information Systems in the Study and Control of Vector-Borne Disease

A geographic information system (GIS) is a

computer software package that captures, stores,

manipulates, queries, displays and analyzes all types

of geographic information. The utility of the GIS is

that it adds a geographical dimension to electronic

data collected and sorted for research purposes. When

related to remote sensing systems on space platforms,

the GIS allows large tracts of land to be analyzed,

integrated and represented in a unified way (Openshaw

1996). As will be shown later in this review, most

vector studies that utilize GIS focus on identifying associations between environmental parameters and vector populations, but the technology is also capable of providing highly accurate estimates of the size of larval habitats and providing images with enough detail to locate and treat the habitats with larvicides. Determining the total land area that requires treatment is essential to estimating the cost of larval control.

Remote sensing has been used to identify surrogates for the meteorological data that have traditionally been used to predict disease vector abundance and mortality rates (Rogers et al. 1996). One common index that is used in this way is the normalized difference vegetation index (NDVI), an estimate of the mean vegetation cover. Factors such as elevation, temperature, rainfall and humidity affect the abundance, longevity and distribution of both vegetation and disease vectors. Therefore, a common postulate for the field of "landscape epidemiology" is that "vegetation, as expressed by landscape elements, can be used to predict the distribution and abundance of certain vector mosquitoes" (Beck et al. 1994). This technology has been utilized in the study of numerous vectors and vector-borne or zoonotic diseases, including trypanosomiasis (Rogers and Randolph 1991), East Coast

fever (Perry et al. 1980), schistosomiasis (Cross et al. 1984), Lyme disease (Glass et al. 1995, Dister et al. 1997, Kitron 1998), Rift Valley fever (Linthicum et al. 1987), and fascioliasis (Malone et al. 1992).

Remote sensing also has been used in numerous studies of malaria. Beck et al. (1994) used images from the digital LANDSAT Thematic Mapper (TM) to estimate the risk of malaria in forty villages in Chiapas, Mexico. They detected a high risk of disease associated with two landscape elements: transitional swamp and unimproved pasture. Using these two elements as part of a predictive model, they were able to identify the villages with higher disease risks on the basis of the abundance of the primary vector, *An. albimanus* Wiedemann, with an accuracy of 90%. From a biological perspective, the association of these landscape elements with high mosquito production is logical. The livestock in the pastures provide the preferred blood meal for the vector, thus contributing to higher populations. The transitional swamp habitat provides large areas suitable for oviposition and larval development. Although other habitat types were identified by TM imaging, including mangrove forests, secondary forest, riparian vegetation, improved pasture, banana plantation, burned fields, urban and inland water, only unimproved pastures and transitional swamps contributed significantly to the

disease prediction model. The authors of this study suggested that their technique of assessing risk through remote sensing could be applied to other vector-borne diseases in areas where the landscape elements critical to vector survival are known and where these elements can be remotely detected.

In another similar study, Roberts and others (1996) used satellite data to predict malaria vector distribution in Belize. They showed that increasing altitude and the presence of filamentous algae in sun-exposed pools were critical environmental determinants for the presence of *An. pseudopunctipennis* Theobald larvae during the dry season. In fact, these authors were able to identify suitable habitats that allowed them to collect *An. darlingi* Root in Belize for the first time since 1946. They suggested that government programs in that country could sustain acceptable levels of control by combining malaria surveillance and vector ecology data with remote sensing and GIS to target the application of insecticides and other malaria control resources.

Remote sensing also has been used in California to predict which rice fields will have the highest production of *An. freeborni* larvae nearly two months before the peak larval density occurs (Roberts and Rodriguez 1994). Fields in early stages of plant development that were near livestock pastures were the

most likely to have high larval densities. The study predicted which fields would have the highest larval density with nearly 90% accuracy. The same authors also noted that certain plant types contributed positively or negatively to the presence of some mosquito larvae and that some of the larger vegetation units were detectable by remote sensing.

Remote sensing and GIS are powerful tools for placing vector occurrence and abundance in a precise geographical context. This type of information can be used to estimate risk, predict vector abundance, and identify habitats that are likely to harbor vector populations. In addition, this technology can become an integral part of the decision-making process for public health. Remotely sensed images can be analyzed to determine the size and location of vector habitats, thus allowing an estimate of costs and providing vector control teams with accurate maps for targeting control efforts.

Cost Analysis and Decision-Making in the Control of Malaria

Choosing between several potential malaria control measures is an important aspect of public health in malarious areas. The decision-making process is affected by numerous factors, including the relative efficacy of the different measures, public

40

relations, politics, environmental issues, drug or pesticide availability, historical use of anti-malarial techniques, cultural aspects and local technical capabilities. From the perspective of program administration, one of the most important determinants in the selection of a control measure is cost. In fact, cost may be considered the single most important issue, yet the lack of information on the costs and effects of many interventions, the small number of published cost-effectiveness studies, and problems with comparing disparate studies render many policies uninformed and result in uninformed decision making (Goodman et al. 1999). The need for cost-effectiveness studies and cost comparisons of appropriate measures is obvious. The World Health Organization's Roll-Back-Malaria program is highly dependent on cost analyses, but that information is often lacking (Goodman and Mills 1999).

More recently, some cost studies of malaria control have been completed, but most have concerned the use of insecticide-treated bed nets. Gonzalez et al. (2000) demonstrated that any of three chemoprophylaxis regimens using combinations of primaqune and iron supplements were less costly than

malaria control measures that rely on clinical case management. In Thailand, the cost-effectiveness of lambdacyhalothrin-treated bed nets was compared to that of residual sprays with DDT; impregnated bed nets were considered most cost-effective (Butraporn 1999). A similar study in Vietnam also determined that treatment of bed nets was more cost-effective than residual spraying, but only if existing nets were treated; purchase of new nets caused the cost to exceed that of spraying (Verle et al. 1999). In sub-Saharan Africa, a cost-effectiveness study of treating existing bed nets, treating and providing nets, residual sprays, chemoprophylaxis for children, intermittent treatment of pregnant women and, and improvement of case management was performed by calculating the cost per disability adjusted life-year (DALY) averted for each method. This unique use of the DALY compared populations without interventions in which life-years were adjusted for presence of disease to populations with interventions in which life-years were unadjusted. Interventions that resulted in different life-years between the adjusted and unadjusted estimates were considered to be DALY-averting. Case-management improvement was considered

to be the most cost-effective, but was still
unaffordable for very-low-income countries (Goodman et
al. 1999). In contrast, White (1999) contended that
house spraying continued to be the most cost-effective
means of malaria prevention and that the economics and
sustainability of insecticide-treated bed nets was
still unproven. One of the concerns about treated bed
nets is the possibility that protecting young children
might actually increase the mortality and morbidity in
older children by delaying the acquisition of
functional immunity (Guyatt et al. 1999). Coleman et
al. (1999) contended that the cost per DALY averted in
places where such malaria "rebound" occurs is a
function of the age at which the children acquire
malaria and the rate of "rebound" in the population.
They concluded that, if rebound occurs in the three to
six year age range, then the use of bed nets is no
longer DALY-averting if the rebound rate exceeds 11%.
In addition, the cost-effectiveness of treated bed
nets exceeds $150.00/DALY-averted if the rate of
rebound exceeds 2.5%.

Cost-comparisons and cost-effectiveness
studies on the control of malaria in the ROK are
nearly non-existent. An unpublished analysis by a

U.S. Army epidemiology team in 1996 concluded that the
cost and difficulty of implementing chemoprophylaxis
for at-risk Army personnel was excessive, especially
given the low number of cases and the non-fatal nature
of the disease (Dr. Stephen Craig, Walter Reed Army
Institute of Research, personal communication).
Instead, the team recommended the use of personal
protective measures (PPM) including the use of
military-issued DEET topical repellent, permethrin-
treated uniforms with sleeves rolled down, and
insecticide-treated bed nets. Subsequent analysis,
however, recommended the limited use of chloroquine
chemoprophylaxis in high-risk areas north of the Imjim
River. The decision to use chemoprophylaxis was
based, in part, on the high levels of non-compliance
in the use of PPM and the large number of transient
personnel, like truck drivers, tank crews and
personnel in training camps. These people were from
diverse commands and areas within the ROK, and were,
therefore, difficult to reach with appropriate
education and supplies. Numerous people entered the
high-risk areas with little training in PPM, and no
repellents or bed nets (LCOL Brian Feighner, MC USA,
Uniformed Services University of Health Sciences,

personal communication, 7 March 2001).

The re-emergence of malaria in the ROK presents several challenges to decision makers seeking effective and affordable control measures. Case treatment and PPM are necessary parts of any program implemented by the U.S. military. Other potential methods to be considered include space sprays for adult mosquitoes, larvicides and chemoprophylaxis. The use of space sprays, like ultra-low volume (ULV) applications, would probably be ineffective in environments like that of the ROK. Such sprays are usually considered temporary and would provide little protection unless nightly applications were used. Even then, the large area of the larval habitats with the resultant nightly emergence of new mosquitoes and the long activity times of the vector would certainly reduce the effectiveness of ULV sprays.

House sprays, or rather the spraying of tents for personnel bivouacked in the field with residual insecticides, might be appropriate for use in the ROK. Strickman et al. (in review) performed residual tent sprays and achieved significant reductions in vector numbers, despite the previously-mentioned report by Chow (1973) that densities of *An. sinensis* were unaffected by residual DDT sprays. However, the exophillic nature of this vector

could decrease the effectiveness of this method. In addition, during the malaria season, military personnel spend a great deal of time outside at night playing sports, patrolling, and undergoing training. Indoor sprays would have little benefit in these situations.

The two malaria control methods that have been considered for the protection of U.S. personnel in the ROK are larviciding and chemoprophylaxis. The costs of implementing these two methods, however, are unknown. The cost of chemoprophylaxis is an important factor in comparing alternatives and can be estimated from the cost of the medicine and the number of personnel requiring protection. The cost of larviciding is a function of the size of the larval habitats and the cost of treatment per unit area.

The study comprising this dissertation utilized remote-sensing to locate and determine the size of various mosquito habitats within a defined buffer-zone around two U.S. bases in the ROK. This data was used to estimate the cost of applying larvicides to the area and to compare with the estimated cost of providing chemoprophylaxis to all active-duty personnel on both bases. This cost comparison was performed to aid in the decision-making process involved in selecting optimal malaria control measures for

the protection of U.S. Army personnel in the ROK.

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68

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CHAPTER 2

ENVIRONMENTAL FACTORS ASSOCIATED WITH LARVAL HABITATS OF MALARIA VECTORS IN NORTHERN KYUNGGI PROVINCE, REPUBLIC OF KOREA

For: Journal of the American Mosquito Control Association

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5 This manuscript reports original research and does not necessarily
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ENVIRONMENTAL FACTORS ASSOCIATED WITH LARVAL HABITATS OF MALARIA VECTORS IN NORTHERN KYUNGGI PROVINCE, REPUBLIC OF KOREA

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ABSTRACT. The larval habitats of malaria vectors near the Demilitarized Zone of the Republic of Korea (ROK) were sampled from June through September, 2000, to determine larval abundance and to identify environmental factors associated with high larval density. Six primary habitats were identified: rice paddies, irrigation ditches, drainage ditches, stream pools, irrigation pools and swamps. Most habitats harbored similar densities of larvae until August and September, when population densities in rice paddies declined and those in irrigation pools increased. The primary vector in the ROK, *Anopheles sinensis* (Wiedemann) was tolerant of a wide range of values for environmental factors, including pH, total dissolved solids, percent surface coverage with floating vegetation, and nitrate/phosphate concentrations. No environmental factor or combination of factors were found that were predictive of high larval densities. This study suggests that *Anopheles* larvae are capable of developing in a wide range of

stagnant, freshwater habitats in northern Kyunggi Province, ROK.

INTRODUCTION After an absence of more than ten years, malaria (*Plasmodium vivax*) has re-emerged in the Republic of Korea (ROK) (Feighner et al., 1998). The number of detected cases grew from one in 1993 to 3,719 in 1999. Of all cases diagnosed in the ROK through 1999, 83 (2.23%) cases occurred in American military personnel or Korean augmentees to the U.S. Army. As of December, 2000, sixteen confirmed cases occurred in American personnel stationed in the ROK for that year alone (personal communication, Preventive Medicine Directorate, 18th MEDCOM). To date, the focus of the disease has been just south of the Korean Demilitarized Zone (DMZ) that separates North and South Korea where large populations of military personnel, both American and Korean, are stationed.

The strain of *P. vivax* transmitted in the ROK is well adapted to a temperate climate and demonstrates both short and long incubation periods. Long incubation periods of greater than six months increase the risk of returning soldiers re-introducing malaria to the continental United States, making malaria control in Korea even more important (Walter Reed Army Institute of Research, 1999).

Until recently, malaria prevention policies for American soldiers in high-risk areas of the ROK relied

exclusively on vector control and personal protective measures. However, Strickman and others (in review) reported acceptable vector and disease control in areas treated with an ultra-low volume adulticide and residual tent-sprays. U.S. malaria policy was modified in 1999 by placing more than 6,000 American soldiers stationed north of the Imjim River on chloroquine/primaquine chemoprophylaxis. Although these drugs are still effective against the strain of *P.vivax* in the ROK, there are some concerns about relying on this method as the primary means of malaria control. For instance, soldiers stationed in areas south of the Imjim River often train for several days in training camps considered to be high-risk areas north of the river. During 1999, soldiers training in "high-risk" areas were placed on chemoprophylaxis prior to the exercise until the end of the malaria season in October. During the 2000 malaria season, only soldiers residing north of the Imjim River were placed on chemoprophylaxis. Soldiers entering these areas only for training depended solely on the use of personal protective measures (PPM), including permethrin treated uniforms, proper wearing of the uniform (pants tucked into the boots and shirt sleeves rolled down), and topical repellents.

In addition to PPM, an area-based control method might provide better malaria control in these areas with transient

military populations. Strickman and others (2000) suggested that larviciding in mosquito habitats around American installations might be a viable control method if such efforts were coordinated with Korean civilian authorities. One advantage of larviciding would be that protection could be conferred on all soldiers in the high-risk areas, regardless of their length of exposure. The cost of such a larviciding program, however, is unknown and would require significant information on the bionomics of vector larvae in the malaria endemic areas. In addition to the coordination of a larviciding program with the surrounding Korean community, cost would be a major consideration. Cost comparisons with other control methods, especially chemoprophylaxis, would be essential.

The cost of larviciding is a function of the size of the larval habitats and the cost of treatment per unit area. Before the size and location of larval habitats requiring treatments can be determined, however, three questions about the local vectors need to be answered.

(1) What larval habitats occur in the high-risk area?

(2) What mosquito larvae occur in the local habitats?

(3) Which mosquito species are proven malaria vectors?

This paper describes efforts to answer the above questions and to develop a quick and reliable means of surveying habitats requiring larvicide treatments.

Successful larval control requires the ability to identify larval habitats and to distinguish between sites with high and low vector populations in a timely manner (Wood et al. 1991). Such a surveillance system is necessary due to the limited manpower available for larval surveillance in the extensive rice paddies and irrigation ditches throughout the ROK. One approach to vector surveillance is to identify key environmental factors that predict the presence of vector populations, then to use these factors as markers to predict the presence of significant larval densities either in an indirect survey or in a remote sensing system. Many such environmental factors have been identified for a variety of vectors. For example, throughout much of its extensive range, *Anopheles pseudopunctipennis* Theobald was associated with green filamentous algae and aquatic vegetation in sunlit freshwater stream pools (Manguin et al. 1996a). In southern Mexico, high *An. albimanus* Wiedemann populations were linked to flooded, unmanaged pastures at an elevation below 25 meters (Rodriguez et al., 1996). A similar but positive association between altitude and the density of *An.*

pseudopunctipennis was noted as well as positive associations with filamentous algae and the presence of the plant *Heteranthera spp.* during the dry season (Savage et al. 1990). In Venezuela, *An. aquasalis* Curry was collected most often in seasonal, brackish mangrove swamps and larval populations varied with the water salinity; whereas, *An. oswaldoi* Peryassu was associated with permanent freshwater habitats (Grillet, 2000). In Belize, the habitats of *An. darlingi* Root were characterized as being river margins with patches of floating debris (Manguin et al., 1996b). Vector populations also have been associated with pH and percent tree cover (Rejmankova et al., 1998).

Researchers have developed models to predict the presence or abundance of vector populations based on environmental factors using logistic regression (Savage et al., 1990), multivariate regression (Rodriguez et al., 1996; Moncayo et al., 2000; Grillet, 2000) and global information systems techniques (Roberts et al., 1999; Roberts and Rodriguez, 1994). Models of this type have the potential to improve the efficiency of malaria vector control and surveillance programs in the ROK, but few if any have been developed for the indigenous malaria vectors.

Malaria vectors in the Republic of Korea

In a review of Korea's arthropods of public health
importance, Chow (1973) mentioned only two potential malaria
vectors: *An. sinensis* (Wiedemann) and *An. yatsushiroensis*
Miyazaki. Of these two species, Chow considered the former
to be the most important vector. This conclusion has
generally been considered correct by subsequent authors, but
questions about the taxonomy of *An. sinensis* and others in
the *'hyrcanus'* group have caused significant confusion. In
particular the difficulty of distinguishing *An. sinensis*
from *An. lesteri* Baisas and Hu is of some importance because
the vector potential of the latter is unknown. Tanaka and
others (1979) considered *An. lesteri* to be the probable
primary vector of malaria in Japan rather than *An. sinensis*,
but little work on this species has been performed on the
Korean peninsula.

In a survey of malaria vectors in Kyonbuk, Korea (Joo
and Kang, 1992), *An. sinensis* was the only species
considered, even though it is generally considered to be
strongly zoophilic. Ree and others (1973) reported only
three *Anopheles* species caught in light traps (*An. sinensis,
An. sineroides* Yamada, and *An. yatsushiroensis*) with *An.
sinensis* comprised 95% of the anophelines and 18% of the

total mosquito population. Strickman and others (1999)
caught the same three species using light traps in the
northern ROK, but also caught one specimen identified as *An.
lesteri*. In that study, larval surveillance indicated
densities of from 0.02 to 3.1 larvae/dip in rice paddies
surrounding a military training area with a probable history
of malaria transmission. A later collection (Strickman et
al., 2000) caught many more adult *An. lesteri* and *An.
yatsushiroensis*, none of which were positive by Enzyme
Linked Immunosorbent Assay (ELISA) for *P. vivax*
circumsporozoite protein. In fact, only two out of 2,376
An. sinensis tested for malaria infection were positive.
Therefore, while *An. sinensis* is not considered to be an
efficient vector, its relative abundance and the detection
of *P. vivax* antigen only in this species implicates this
mosquito as the primary vector in the ROK.

This study investigated the distribution of *Anopheles*
larvae among various larval habitats and attempted to
identify environmental factors that could be used to locate
efficiently the sites producing the most malaria vectors.

MATERIALS AND METHODS The study was performed from June
through September, 2000, in the northwest part of Kyunggi
Province, ROK, about 60 km north of Seoul, and near the DMZ

in the vicinity of Munsan. This area was characterized by a temperate climate with four distinct seasons. Average temperatures ranged from -7 to 2°C in the winter and up to 20 to 29° C in the summer. Monsoons usually affect the area, with the heaviest average rainfall by season occurring in the summer months (mean = 258 mm). The summer of 2000 yielded unusually low rainfall in the ROK; however, typhoon-associated rainfall was significant and provided enough precipitation to flood many of the study sites, especially the streamside pools. Sample sites were concentrated in the areas surrounding the Camp (CP) Casey complex and CP Greaves (both U.S. military bases). The CP Casey complex consisted of three military facilities that included the contiguous CPs Castle and Hovey. The complex was transected by a large, permanent stream that was prone to flooding after heavy rainfall. The towns of Tongduchon and Tokori surrounded the CP Casey complex on three sides, but rice cultivation continued at the interfaces between the camps and villages.

The CP Greaves area was smaller and located in a rural environment. A high bluff overlooking the Imjim River bordered the southern perimeter of CP Greaves. Rice farming was extensive on both sides of the river and continued from

the camp into the DMZ. Cattle and goat herds were located near the eastern extension of CP Greaves.

Both areas were characterized by low, wooded hills separated by shallow valleys. The valleys were usually planted in rice, but also supported crops of peppers, corn, onion, ginseng and cabbage. Other than the one large stream transecting CP Casey, few natural streams occured in the study areas. Rather, numerous irrigation ditches and ponds were interspersed between the rice paddies, providing a variety of potential larval habitats.

Six habitats were identified for sampling purposes: rice paddies, irrigation ditches, drainage ditches, swamps, stream pools, and irrigation pools. The rice paddies were typically small (less than 1,300 m^2) and were bordered by dikes ranging in height from 0.3 to 2.0 meters. Early in the season, the dikes were typically mowed and devoid of tall vegetation; however, the degree to which the borders were maintained varied, and some became overgrown later in the season. The paddies were transplanted with seedlings in May through early June. Irrigation or flooding of the rice paddies was inconsistent so that some were flooded while others at the same stage of plant development were not. Irrigation ditches ranged in size from small troughs 0.3 meters deep to large canals up to 2 meters wide and 1 meter

deep. Drainage ditches also varied in size and were frequently lined with concrete or stone. Stream pools were observed only on CP Casey. Pools ranged in depth from a few centimeters up to one meter, and in surface area from less than 1 m^2 to about 100 m^2. They often supported heavy algal mats and had sand or mud bottoms; most were surrounded by large stones or concrete sides. Irrigation ponds were located near the rice paddies and provided water for irrigation. The ponds were usually about 1.5 meters deep (range: 800 mm-2,100 mm) with steep sides. Early in the season, few larvae were found in these sites, but by August, large populations of larvae were observed among the algae growing along the edge. Swamps were not common so only two were sampled during this study. The swamps supported a wide range of vegetation and were always flooded. The two swamps were included only for a general survey of the species present and were not included in statistical analysis.

Data collection Initially, 60 study sites were identified, sampled, and located with a Garmin III® hand-held global positioning satellite (GPS) unit. The GPS positions were collected for Geographic Information Systems (GIS) work to determine the size of larval habitats in high-risk areas. Thirty sites were located around each of the two camps,

Greaves and Casey. As the season progressed, other sites became available and were added to the study for a total of 93 sites: 50 rice paddies, 13 stream pools, 12 irrigation ditches, 11 drainage ditches, 5 irrigation pools and 2 swamps.

Larval sampling was performed with a standard plastic larval dipper around the perimeter of each study site. The circumference of each site was measured then divided by thirty so that thirty dips could be equally spaced around the site. Initially, all anopheline larvae and a sample of culicine larvae were removed with pipettes and stored in plastic bags for transport to the central laboratory at CP Casey. When *Anopheles* populations were excessive, approximately 10% of the larvae were taken for rearing. The plastic bags were filled with water from the study site then stored in a plastic ice chest during transport of the larvae to the laboratory for rearing. All collections were performed between 0700 and 1200 hours. With the exception of those added later in the study, each habitat was sampled once each month from June through September. After each additional site was added, it was sampled monthly through September. A value of "0" was recorded for sites that were dry, flooded with running water, or otherwise incapable of

supporting larval populations, as well as for suitable sites with no larvae in 30 dips.

To investigate the distribution of *Anopheles* larvae within the rice paddy habitat, five paddies were chosen for a smaller study. A long, rigid plastic pole was attached to one of the dippers, allowing samples to be taken approximately two meters into the paddy. Paired samples were taken from the perimeter, one within 0.3 meters and the other two meters from the edge. Thirty paired samples were taken in each of the five rice paddies. Sampling error is probable because dips from the interior of the paddy were more difficult to obtain without losing some of the water from the dipper. However, the same dipper was used to obtain edge and interior dips and great care was taken to standardize the sampling technique for interior and perimeter dips. Perimeter sampling was required because we were unable to obtain permission from the landowners to wade into the paddies. In order to geographically visualize the distribution of anopheline larvae within the rice paddy habitat, the data from the paired samples were imported into ArcView® GIS 3.2 (Environmental Systems Research Institute, Inc., Redlands, CA) running ArcView® Spatial Analyst 2.0 and an extension named SERDP. The SERDP extension was developed by the Center for Medical and Veterinary Entomology, USDA,

Gainesville Florida to simplify geostatistical data analysis

and was downloaded from their website at

http://cmave.usda.ufl.edu. Inverse distance weighting was used

to produce larval population geographical contours within

the outer 2-meter margin of the four rice paddies where

larvae were found in the interior dips. The contours in

each image indicate an area with a 0.5 probability of

containing 85% of the larvae collected in the entire paddy.

In June and July, water analysis was conducted on one

of the thirty dips from each site. Nitrate and phosphate

concentrations were determined with Hach® Pocket Colorimeter

Systems; total dissolved solids (TDS) and pH were both

determined with Hach® Pocket Pal testers.

Percent of water surface covered by duckweed (*Lemna*

spp.) was estimated; the same was performed for the percent

surface area covered by algae of all types. Three observers

made independent visual estimates of the duckweed or algal

cover and the mean of the three estimates was recorded.

Rearing procedures Because it is impossible to distinguish

the species of Korean *Anopheles* by larval characteristics,

the larvae were reared to the adult stage for specific

identification. Mosquito rearing was performed in an air-

conditioned water-testing laboratory at CP Casey. A plastic

drape was placed over a counter top and the interior was warmed continuously with two 100-watt incandescent light bulbs. The larvae were transferred to 16-oz. plastic cups filled with tap water that had been allowed to sit overnight to reduce chlorine residuals. Larvae were fed finely ground Tetramin® fish food. When they reached the fourth stage, each larva was moved to an individual plastic rearing vial. The fourth-stage and pupal exuviae were removed and placed in a one-quarter dram glass vial with a rubber stopper in 80% ethanol for taxonomic study. The adults were preserved according to procedures of Belkin and others (1965), then identified to species according to Lee (1998). Voucher specimens of adults and associated exuviae were archived at the National Museum of Natural History, Smithsonian Institution, Washington, DC.

Statistical analyses Mean and median numbers of *Anopheles* densities (number of larvae/30 dips) were determined with the UNIVARIATE procedure in SAS (SAS Institute, 2000). Medians were compared using the Kruskal-Wallis test, but data with similar coefficients of variation were compared with the Tukey's Honestly Significant Differerence test. Similar analyses were used to compare environmental parameters by habitat, with both types of ditches combined

into one category and both types of pools similarly combined.

In order to develop a predictive model for sites with high densities of *Anopheles* larvae, each environmental parameter was dichotomized at the median and at the 75th percentile to allow analysis by logistic regression. The LOGISTIC procedure was used to perform stepwise logistic regression modeling of the probability of a site being in the top quartile of larval density as a function of water quality and amount of surface covered by vegetation.

RESULTS Of the fourteen putative species collected during the study period, only three were anophelines (Table 1). These mosquitoes were all identified as adult specimens using characteristics described in Tanaka et al. (1979) and Lee (1998). Adult characteristics used to separate *An. sinensis* and *An. lesteri* may not be reliable, so the relative numbers these two species must be considered with some caution. Nevertheless, the majority of *Anopheles* mosquitoes reared to adulthood (81%) appeared to be *An. sinensis,* with *An. lesteri* representing only 18.8%. Only three specimens of *An. yatsushiroensis* were reared to the adult stage during the study, representing less than 1% of the *Anopheles*. Other species that were identified in

significant numbers were *Aedes vexans* (Theobald) and *Culex pipiens* Linnaeus.

Anopheles sinensis were collected in all six of the habitats sampled (Table 1). *Anopheles lesteri* was only obtained from rice paddies, stream pools and irrigation ditches, but this finding may be due to the small number of specimens reared to the adult stage. Densities of *Anopheles* larvae for most habitats were similar in June and July (Table 2). A trend toward greater larval densities in ponds and stream pools started in July and this trend eventually led to significantly greater densities in these habitats in August and September when compared to ditches and rice paddies. The median number of larvae per dip in rice paddies was never very large, though as many as 50 larvae were collected in some dips. In other words, distributions of larvae were highly clustered within rice fields.

In September, many rice paddies were drained in preparation for harvesting, resulting in the elimination of larval habitats; however, very large larval densities developed in the pools, especially irrigation lagoons contiguous to the drained rice paddies. The pools supported the highest larval densities observed during the four-month study.

Tables 3, 4, 5, 6, and 7 describe environmental factors for each of the habitats. The factors overlapped in their ranges and no statistically significant differences were noted between habitats, with the exception of percent surface covered by floating duckweed (*Lemna* spp.) Rice paddies had significantly greater coverage with this plant, though percent coverage was not predictive of larval densities using logistic regression techniques ($p = 0.45$). The similarity in water quality parameters is perhaps not surprising given that several of the habitats are interconnected with a complex irrigation system that moves water from streams, rivers and wells to irrigation ditches to rice paddies then back again. In some paddies, this movement was continuous and resulted in a slow movement of water through the paddies. Other paddies were more stagnant and prone to intermittent drying. Environmental factors were also similar for infested and non-infested sites (i.e. fields with and without detectable larval populations). The range of each factor is reported for infested and non-infested paddies in Table 8. The results indicate that anopheline larvae in the ROK can tolerate significant variations in water quality and plant coverage.

Stepwise logistic regression analysis revealed no factors that were predictive of high larval densities ($p >$

0.05). The most likely candidate for predicting highly infested paddies was percent duckweed coverage adjusted for nitrate and phosphate concentrations in July ($p = 0.06$), but the overall model was non-significant ($p = 0.24$).

The percent of each habitat in which at least one larva was detected varied monthly. For rice paddies, peak percent infestation (77%) was observed in July, but fell to 20% in September when most of the paddies were drained. In contrast, none of the irrigation pools yielded anopheline larvae in June, but by September, all of them supported heavy larval populations. Larval populations in stream pools and drainage ditches were variable, as they were prone to heavy flooding from typhoon-associated rains.

The distribution of *Anopheles* within individual rice paddies was not uniform but was concentrated around the margins of the paddies. Three out of the five paddies surveyed had a rate ratio (number of dips with at least one larva in a dip/number of dips with no larvae) between 1.8 and 2.0; both of the other paddies had much larger ratios. The ratios of the number of larvae caught in dips near the perimeter divided by the number caught in the same number of dips toward the center of the paddy ranged from a minimum of 1.7 to an infinite ratio in which all of the larvae were collected at the perimeter. The figure illustrates that the

majority of positive dips were near the edge, and that
generally the greatest number of larvae caught were near the
edge as opposed to the interior of the paddy. Of 28
positive sites within the paddies, 21 were obviously
concentrated along the edges.

DISCUSSION *Anopheles* larvae in the ROK tolerate a wide range of the environmental factors investigated during this study. Due to the ability of the larvae to thrive in such a variety of conditions and habitats, none of the water quality or vegetation indices recorded in this study were reliable predictors of high larval density. This conclusion is consistent with previous research by Ikomoto and Sakaki (1979), who found a lack of consistent correlation between *An. sinensis* population density and the pH and temperature of rice paddy water. However, there was a positive correlation between the number of immatures and the NH_4-N concentration in that study that was not reflected in the nitrate concentration analysis of our work.

In our study, larvae were collected from a variety of habitats and, at least early in the growing season, showed limited identifiable preference for habitat type. Rather, the larvae seemed capable of exploiting most stagnant or slowly moving water habitats in the study area. Nearly 26% of the sites sampled in July, however, did not support detectable larval populations; the reason for the lack of larval populations in these habitats is unknown but may be related to chemical fertilizers and pesticides applied to the paddies at irregular intervals. Nevertheless, assuming that most of the *Anopheles* in the

area were *An. sinensis*, we can conclude that this malaria vector is capable of development in areas with or without floating vegetation and in water with a broad range of pH, TDS, and nitrate/phosphate concentrations. Other environmental factors not investigated during this study may, however, serve as limiting factors. It is important to note that the sampling method used during this study was designed for study of large habitats that could be identified and analyzed remotely. A sampling technique that analyzed at the level of a microhabitat (ie. algal mats vs. overhanging vegetation) might be more likely to identify environmental parameters that are predictive of vector abundance at a different scale.

The relative proportion of *Anopheles* species reared to the adult stage in our study differs from the proportion of species attracted to adult traps during a contemporary study performed in the same study site by Burkett and others (in review). In particular, very few *An. yatsushiroensis* were obtained during this larval study, whereas this species comprised nearly 48% of *Anopheles* captured with a Shannon trap during the same time in the same location. Several factors may contribute to the inconsistency between the larval and adult studies. There may have been differential mortality in the rearing process that resulted in the death

of most of the *An. yatsushiroensis* larvae. Alternatively, the distribution of this species in rice paddies may be such that perimeter dipping does not reflect the proportion of species present in the rice paddy habitat. This study suggests that *Anopheles* larvae are distributed unevenly in the rice paddies. Finally, there may be another habitat that is exploited preferentially by *An. yatsushiroensis*. Of these three possibilities, the third seems most likely. The adult trapping performed by Burkett and others was done near Camp Greaves, an area that is still plagued with land mines left over from the Korean War. These mines prevented larval sampling in some areas, especially in wooded, hilly areas, and along the banks of the Imjim River. Habitats within these areas or habitats within the river may serve as the preferred larval habitats of *An. yatsushiroensis*. Tanaka et al. (1972) report that this species is common in rice fields but that it is more common in hilly or mountainous areas.

The densities of anopheline larvae were similar for most habitats in June and July, but , in September, when the rice paddies were drained, larval densities in the irrigation pools greatly increased. In fact, the greatest larval densities during the entire four months occurred in September in the stream pools and irrigation lagoons. A larval control program directed toward pools and ditches

late in the season would eliminate the heaviest concentrations of the vector population, though the relative size of the rice paddy habitat would still make these sites major contributors to overall vector numbers.

Several aspects of the biology of malaria vectors in the ROK need to be addressed in future studies. First, the larval habitat of An. *yatsushiroensis* should be identified. This species may play some role as a malaria vector and thus might be important in the epidemiology and control of this disease. Second, other environmental factors should be investigated as possible predictors of the presence and abundance of *Anopheles* larvae. One factor that appears to have a substantial impact on larval density that was not addressed in this study is the pattern of flooding and draining in the rice paddies. Many paddies were drained in the middle of the growing season, perhaps accidentally. More attention should be directed to the effect of flooding and draining on species composition and abundance within the rice paddy habitat. Third, the taxonomy of *Anopheles* in the ROK must be clarified before specific work on the biology and control of members of this genus can be completed. In particular, the ability to distinguish between An. *lesteri* and An. *sinensis* using adult morphological characteristics is questionable. At present, numbers of specimens for these

species from this study must are tentative until further systematic work to validate species identification is completed.

This paper reports the first part of a larger study using remote sensing to determine the size and quality of *Anopheles* larval habitats within the flight range around two U.S. military bases in the ROK. This information will be used to estimate the cost of a larviciding program and to compare that estimate to the cost of providing chemoprophylaxis. It should be noted that the U.S. government is not considering any unilateral larviciding efforts, and would implement such a malaria control program only with the support and concurrence of the ROK governmental authorities and local landowners.

This study was conducted as part of the Doctor of

Public Health program at the Uniformed Services University

of Health Sciences.

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Table 1. Mosquito species taken from larval habitats in northern Kyunggi Province, ROK and reared to adults (June - September, 2000).

Species (# specimen) reared to adult stage	Habitats where species was found
An. sinensis(442)	Rice paddy, stream pool, drainage ditch, irrigation ditch, swamp
An. lesteri(42)	Rice paddy, stream pool, irrigation ditch
An. yatsushiroensis(3)	Irrigation pond, drainage ditch
Ae. vexans(277)	Rice paddy, stream pool, drainage ditch, irrigation ditch, irrigation pond, swamp
Ae. albopictus(4)	Discarded tire
Ae. koreicus(3)	Discarded tire

Table 1. (Con't)

Cx. vagans(41)	Rice paddy, steam pool, drainage ditch
Cx. pipiens(79)	Rice paddy, drainage ditch, irrigation ditch, irrigation pond, swamps
Cx. orientalis(36)	Rice paddy, streams pool, drainage ditch
Cx. tritaeniorhyncus(30)	Rice paddy, drainage ditch, irrigation ditch
Cx. mimeticus(7)	Rice paddy, stream pool
Cx. hayashi(3)	Irrigation pond
Cx. bitaeniorhyncus(10)	Irrigation ditch, stream pool
Cx.rubensis(1)	Swamp

Table 2. Median number of larvae per dip (*n, standard deviation, range*) of *Anopheles* mosquitoes collected in larval habitats of northern Kyunggi Province, ROK (June - September, 2000).

Habitat	Month			
	June	July	August	September
Rice paddies[1,2]	0.1 a	0.4 a	0.3 a	0.0 a
	(42, 0.9, 0-5.3)	*(42, 0.9, 0-4.7)*	*(49, 0.4,0-1.9)*	*(41, 0.6, 0-3.0)*
Ditches	0.1 a	0.4 a	0.1 a	0.1 a
	(10, 0.3, 0-0.9)	*(11, 1.8, 0-6.0)*	*(17, 0.3, 0-0.9)*	*(14, 1.4, 0-4.7)*
Pools & ponds	0.0 b	0.7 a	0.4 a	5.0 b
	(8, 0.0, 0-0.1)	*(12, 4.3, 0-14.7)*	*(14, 1.4, 0-3.0)*	*11, 13.1, 0-39.3)*

[1] Median values within a column followed by the same letter are not significantly different (Kruskal-Wallis; p > 0.05).

[2] Irrigation ditches and drainage ditches were combined into one category for purposes of statistical analysis. Stream pools and irrigation ponds also were combined.

Table 3. Percentage of area covered with algae in three larval habitat types of northern Kyunggi Province, Republic of Korea (June-September, 2000)

Habitat	Mean	n	Median[1]	Standard deviation	Range	Coefficient of variation
Rice paddies	7.6	50	0 a	20.1	0-80	2.6
Ditches[2]	18.9	20	0 a	26.9	0-75	1.4
Pools[2]	19.4	15	0 a	32.3	0-100	1.7

[1] Medians followed by the same letter are not significantly different (Kruskal-Wallis test; p = 0.05).

[2] Irrigation ditches and drainage ditches were combined into one category for statistical analysis. Stream pools and irrigation ponds also were combined.

Table 4. Percentage of area covered with *Lemna spp.* in three habitat types of northern Kyunggi Province, Republic of Korea (June-September, 2000)

Habitat	Mean	n	Median[1]	Standard deviation	Range	Coefficient of variation
Rice paddies	40.2	50	30 a	38.4	0-100	1.0
Ditches[2]	6.0	18	0 b	14.1	0-50	2.4
Pools & Ponds[2]	2.0	15	0 b	0.7	0-10	0.4

[1] Medians followed by the same letter are not significantly different (Kruskal-Wallis; p > 0.05).

[2] Irrigation ditches and drainage ditches were combined into one category for statistical analysis. Stream pools and irrigation ponds also were combined.

Table 5. Mean nitrate concentration (mg/L) in three habitat types of northern Kyunggi Province, Republic of Korea (June-September, 2000)

Habitat	Mean[1]	n	Standard deviation	Range	Coefficient of variation
Rice paddies	0.9 a	37	0.8	0-2.8	0.9
Ditches[2]	1.4 a	16	1.1	0-4.0	0.8
Pools & Ponds[2]	1.2 a	12	0.7	0.4-2.6	0.6

[1] Means followed by the same letter are not significantly different (Tukey's HSD; p > 0.05).

[2] Irrigation ditches and drainage ditches were combined into one category for statistical analysis. Stream pools and irrigation ponds also were combined.

Table 6. Phosphate concentration (mg/L) in three habitat types of northern Kyunggi
Province, Republic of Korea (June-September, 2000)

Habitat	Mean[1]	n	Standard deviation	Range	Coefficient of variation
Rice paddies	0.4 a	44	0.5	0.1-2.4	1.25
Ditches[2]	0.7 a	18	0.6	0.1-2.1	0.9
Pools & Ponds[2]	0.3 a	14	0.3	0.0-1.3	1.0

[1] Means followed by the same letter are not significantly different (Tukey's HSD;
p > 0.05)

[2] Irrigation ditches and drainage ditches were combined into one category for
statistical analysis. Stream pools and irrigation ponds also were combined.

Table 6. pH in three habitat types of northern Kyunggi Province, Republic of Korea (June-September, 2000)

Habitat	Mean[1]	n	Standard deviation	Range	Coefficient of variation
Rice paddies	7.5 a	38	1.1	5.7-9.6	0.1
Ditches[2]	7.4 a	16	1.1	6.3-10.0	0.1
Pools & Ponds[2]	8.0 a	14	1.2	6.2-9.6	0.2

[1] Means followed by the same letter are not significantly different (Tukey's HSD; p > 0.05).

[2] Irrigation ditches and drainage ditches were combined into one category for statistical analysis. Stream pools and irrigation ponds also were combined.

Table 7. Total dissolved solids (ppm) in three habitat types of northern Kyunggi

Province, Republic of Korea (June-September, 2000)

Habitat	Mean[1]	n	Standard deviation	Range	Coefficient of variation
Rice paddies	159 a	38	80.5	0-375	0.5
Ditches[2]	200 a	16	78.8	116-438	0.4
Pools & Ponds[2]	178 a	. 14	55.8	106-285	0.3

[1] Means followed by the same letter are not significantly different (Tukeys HSD; p>

0.05).

[2] Irrigation ditches and drainage ditches were combined into one category for

statistical analysis. Stream pools and irrigation ponds also were combined.

Table 8. Mean (range) of environmental variables in study sites with and without *Anopheles* larvae, Kyunggi Province, ROK, (June - September, 2000). No significant differences were noted between infested and non-infested habitats (Kruskal-Wallis; p = 0.05).

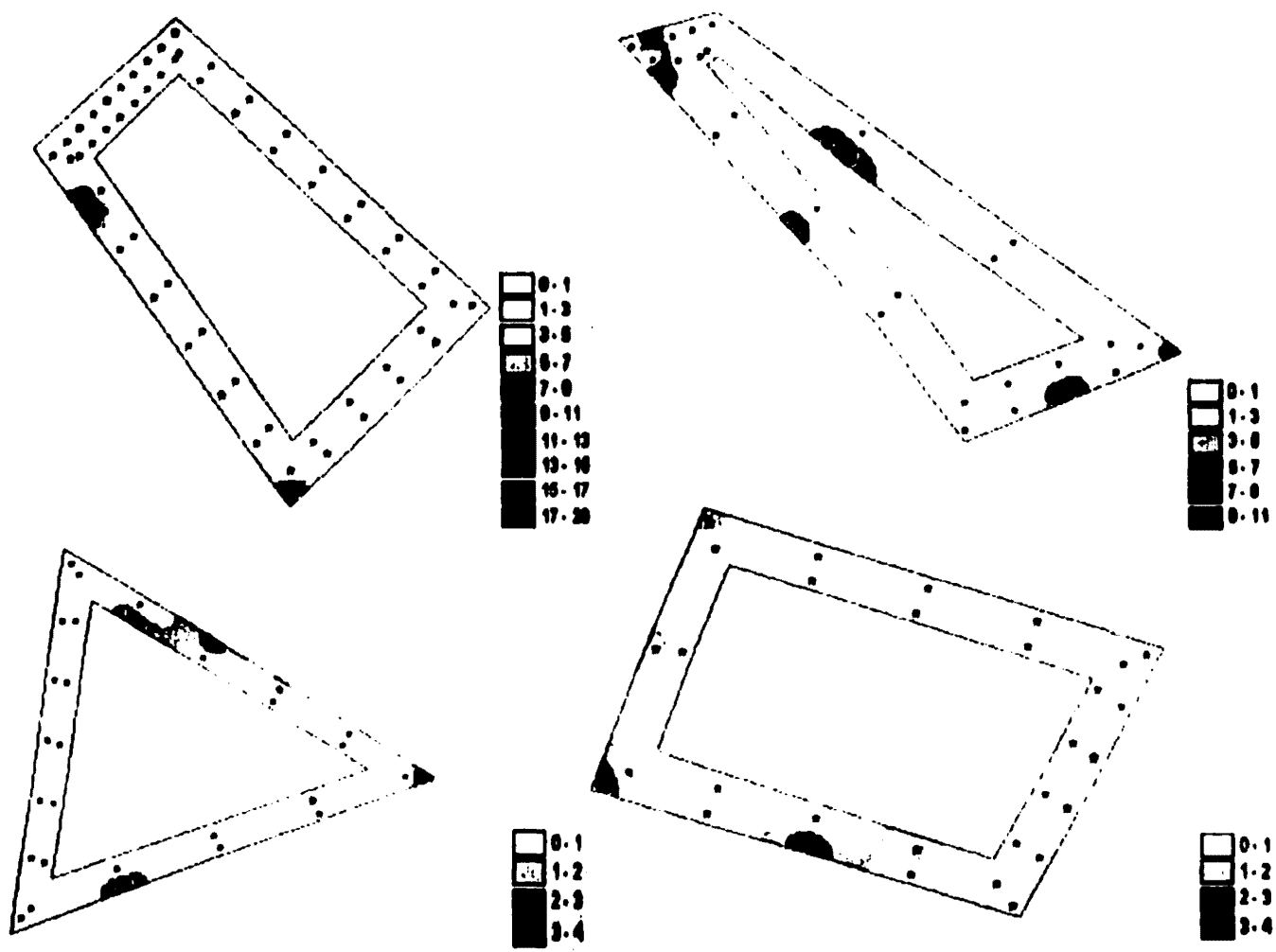| Environmental factor	Larval presence	
	Yes	No
% algal coverage	11.3 (0-80)	13.8 (0-100)
% *Lemna* spp.coverage	31.7 (0-100)	17.3 (0-100)
Nitrates (mg/L)	0.9 (0-2.8)	1.4 (0.1-4.0)
Phosphates (mg/L)	0.4 (0-2.4)	0.4 (0.05-2.05)
pH	7.6 (5.9-9.9)	7.6 (5.7-10)
TDS (ppm)	164.0 (0-375)	182.0 (103-438)

Figure 1. Probabilities of *Anopheles* larval abundance in four rice paddies in Kyunggi Province, ROK (July, 2000).

CHAPTER 3

MOLECULAR AND MORPHOLOGICAL ANALYSES OF ADULT CHARACTERISTICS FOR IDENTIFICATION OF MALARIA VECTORS (DIPTERA:CULICIDAE) IN THE REPUBLIC OF KOREA

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MOLECULAR AND MORPHOLOGICAL ANALYSES OF ADULT
CHARACTERISTICS FOR IDENTIFICATION OF MALARIA VECTORS
(DIPTERA:CULICIDAE) IN THE REPUBLIC OF KOREA

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ABSTRACT

Three methods - RAPD analysis, gene
sequencing, and pupal morphology- were used to
determine the validity of adult morphological
characteristics for separation of the malaria
vectors *Anopheles sinensis* and *An. lesteri*. One
gene sequence (DII) was identical for each putative
species; whereas, another sequence (COII) showed
limited differences that resulted in a grouping of
the individuals that was inconsistent with adult
morphology. Random amplification of polymorphic DNA
(RAPD) analysis suggested that almost all of the
specimens were identical to colonized *An. sinensis*
from China. Overall results suggest that adult
morphological characteristics currently used to
separate these two species in the Republic of Korea
are not reliable. Use of adult morphological
characteristics alone is likely to result in a
significant overestimation of the relative abundance
of *An. lesteri*. One pupal characteristic (the
pattern of pigmentation on the wing sheath) may be
the most reliable morphological feature for

separating these two species. This study demonstrates the advantages of using a multidisciplinary approach to mosquito systematics because key morphological characters being considered for use in a dichotomous key can be validated with molecular techniques.

Key words: *Anopheles sinensis*, *Anopheles lesteri*, RAPD, systematics.

MOLECULAR AND MORPHOLOGICAL ANALYSES OF ADULT CHARACTERISTICS FOR IDENTIFICATION OF MALARIA VECTORS (DIPTERA: CULICIDAE) IN THE REPUBLIC OF KOREA

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The re-emergence of malaria in the Republic of Korea (ROK) generated renewed interest in the systematics of malaria vectors in that country. Black and Munsterman (1996) contended that correct identification of vectors is critical for suppression of vector-borne disease outbreaks. However, as early as 1973, Harrison reported that several of the adult morphological characteristics used to separate *Anopheles sinensis* Wiedemann and *An. lesteri* Baisas and Hu, the presumed vectors in the area, were useless. Later, Strickman et al. (1999) noted the need for clarifying the taxonomic status of the three species of mosquitoes considered to be primary or secondary malaria vectors in the ROK, including the less frequent *An. yatsushiroensis* Mayazaki. The ability to distinguish the two more abundant species, *An. sinensis* and *An. lesteri*, is important because their relative vector statuses, or degree to which

123

they actually transmit malaria in the ROK, are unknown. In addition, there are observed differences in preferred larval habitats (Tanaka et al. 1979).

Anopheles sinensis is considered to be the primary malaria vector in the ROK due to high abundance and the detection of *Plasmodium vivax* sporozoites in the salivary glands of this species (Chow 1973). The vector status of *An. lesteri*, while not well established in the ROK, is well documented in other parts of Southeast Asia and China, especially in the Philipines where it is considered to be a primary vector (Tanaka et al. 1979).

Both *An. sinensis* and *An. lesteri* are members of the 'An. hyrcanus' complex. The taxonomy of this complex is very difficult and some members meet the definition of "sibling species" or species that are reproductively isolated, but appear as a single morphological species even to taxonomic specialists. For species that can be separated only at a certain life stage, accurate identification requires that the immatures be collected and reared to maturity, or mated and reared through another generation. Even with such procedures, morphological characteristics

have not always been sufficient for accurate classification (Black and Munsterman 1996).

Molecular techniques provide new taxonomic tools for species separation and vector incrimination. Numerous molecular studies of species complexes and sibling species have been carried out, including studies of 'quadrimaculatus' (Cornel et al. 1996), 'freeborni-hermsi' (Porter and Collins, 1991), 'minimus' (Bortel et al. 2000) 'japonicus' (Fonseca et al. (unpublished data)), 'gambiae-arabiensis' (Besansky et al. 1997; Lehmann et al. 2000), and 'albitarsis' (Wilkerson et al. 1995).

In this study, we used a combination of molecular and morphological techniques to evaluate the current identification methods for two adult malaria vectors in the ROK.

MATERIALS AND METHODS

The anopheline mosquitoes for this study were collected as larvae from rice paddies and other habitats in Kyunggi Province, ROK, from June through September, 2000. They were reared to the adult stage and females were pinned. Specimens were identified according to adult characteristics as described in Lee (1998). The pupal and fourth-instar exuviae were collected for each specimen and preserved in 80% ethanol. Forty pupal exuviae of each species (based on the associated adult's morphology) were slide mounted and identified according to Ohmori (1959). Three pupal characteristics were used to separate the exuviae -the thickness of the trumpet, the presence or absence of a large spot between the trumpets, and the shape of pigmentation spots on the wing sheaths.

Gene sequencing

Five adult specimens of each morphotype (*An. sinensis* and *An. lesteri* as identified by adult characteristics alone) were used in a gene sequencing study to assess the agreement between the morphological and molecular techniques in the

identification of the two species. We sequenced 549 base pairs of the COII gene, which is reposonsible for production of carboxyl oxidase, then, based on the findings from the COII sequences, selected four specimens that separated by that gene and sequenced 547 base pairs of the DII gene. These genes were selected because they had proven useful in previous genetic studies of mosquito complexes performed at the Walter Reed Biosystematics Unit, where the laboratory phase of this study was performed. The COII primers had the following sequences:

5' - AGTTCATCTCCTTTAATAGAACA (forward)

5' - ACACAAATTTCTGAACATTGACCA (reverse)

The D II primer sequences were:

5' AGTCGTGTTGCTTGATAGTG 3' (forward)

5' CTTGGTCCGTGTTTCAAGAC 3' (reverse

For the amplification, we used 5 ng of genomic DNA in a 50 μl reaction. The final concentrations of the PCR reagents were as follows: 1 x PCR buffer, 300 nM of

each primer, 250 µM of each dNTP, 2 mM MgCl$_2$, and 1.5

units of Taq polymerase (PE Biosystems, Foster City,

CA). The PCR amplifications were preceded by a 5-

minute denaturation at 96 °C. The amplification

consisted of 40 cycles of 40 seconds at 94° C, 4 s at

55 °C, and 60 s at 72 °C. The process ended with a

final extension step of 7 min at 72 °C and storage at

4 °C. Sequences were visualized and scored on a 377

PE Biosystems Automatic Sequencer (PE Biosystems,

Foster City, CA).

Sequences were cleaned, and forward and

reverse sequences assembled into a consensus with

Sequencher 3.0 (Gene Codes Corp., Ann Arbor, MI).

Alignment was performed manually.

PCR-RAPD analyses

Five specimens of the putative species *An.*

lesteri (identified by adult characteristics alone)

and four *An. sinensis* were selected for RAPD analysis

to investigate the relationship between the wild-

caught specimens. For comparison purposes, *An.*

sinensis from an established colony in Shanghai,

Peoples Republic of China, also were used. In

128

addition, one specimen that had both adult and pupal morphological characteristics of *An. lesteri* was selected for study. The Chinese specimens were preserved in 70% ethanol until DNA was extracted; all others were stored dry. We extracted DNA using a phenol/chloroform method (Fonseca et al. 2000). Randomly amplified polymorphic DNA (RAPD) analysis was performed on the selected specimens. Each RAPD-PCR reaction of 25 μl contained 15.6 μl of distilled water, 11 mM Tris-HCL buffer (pH 8.3), 50 mM KCl, 1.9 mM MgCl$_2$, 0.1 mg/ml BSA, 0.1 mM each of dATP, dTTP, dCTP, and dGTP, 0.5 - 1.5 units of Taq polymerase, 0.2 - 4.0 ng/μl template DNA, and 0.24 pmol/μl primer. Twenty randomly-selected primers were screened and twelve were used as discriminators. Table 1 lists the sequences of each primer used in the study.

DNA amplification was performed on a PE Biosystems 480 Thermocycler (PE Biosystems, Foster City, CA). PCR amplifications were preceded by five minutes of denaturation at 96°-C. The cycling program used was 40 cycles of 94°-C (1 minute), 35°-C (1 minute) and 72°-C (2 minutes). Final incubation was performed at 72°-C for 2 minutes followed by cooling to 4°-C. Amplification products were analyzed by

electrophoresis in 1.5% agarose gels with TAE buffer and ethidium bromide. Phi-X vector cut with HAE III and HindII (Sigma Inc., St. Louis, MO) was used as amolecular marker. Each gel was individually placed on a black light table and photographed, converted to a negative image, and copied to a transparency. The transparency was placed over a grid system to facilitate the identification of bands. For each column, a value of '1' was allocated to a block containing a DNA band and a value of '0' allocated to a block without the same band. The Nei-Li coefficient for every specimen-to-specimen comparison was calculated (PAUP, Version 4.0b6). The median distances for each comparison were compared using the non-parametric Kruskal-Wallis test (SAS 1993).

Of 228 anopheline females reared to the adult stage, 179 (79%) were *An. sinensis* and 46 (20%) were *An. lesteri* as determined by adult morphology alone. Only three *An. yatsushiroensis* were reared to the adult stage. Of 40 specimens identified morphologically as *An. sinensis* at the adult stage, all of the associated pupal exuviae had pupal characteristics described for this species - uniform thickness of the trumpet, no prominent pigmentation spots on the dorsal plate between the trumpets, and rows of pigmentation spots on the wing sheaths.

Pupal characteristics for *An. lesteri* are described as "trumpet expanded -a pair of dark markings on the dorsal plate between both trumpets…wing sheath with yellowish to dark brown patterns along the veins, usually adding dark brown cross stripes (Ohmori 1959)." Of 40 specimens identified morphologically as *An. lesteri* at the adult stage, none of the associated pupal exuviae had dark markings on the dorsal plate and only one had observably expanded trumpets. However, three specimens had dark patterns on the wing

sheath that were easily distinguished from the spotted
pattern of *An. sinensis*. Images of these markings are
submitted as Appendix A.

Gene sequencing data

No mutations were observed in the DII gene,
but a total of eight mutations was observed in the
COII gene across the ten specimens examined (Figure
1). Three specimens had seven mutations that
separated them from all other specimens. However,
this separation was inconsistent with adult
identifications because each sub-group contained
representatives of each putative species. Sequences
are included in this dissertation as Appendix B and
will be submitted to GenBank upon submission of a
manuscript.

RAPD-PCR Analysis

Analysis by RAPD also failed to validate species
identification by adult characteristics. Only the
specimen with *An. lesteri* characteristics at the pupal
stage separated consistently from the wild-caught

Korean specimens and the Chinese colonized specimens. The Nei-Li distance for all comparisons is reported in Table 2. The median Nei-Li distance estimates between '*lesteri*' and '*sinensis*' specimens (Table 3) were not significantly different from the intra-group distance estimates for each species, suggesting that these two groups are not distinct species. However, the one specimen identified as *An. lesteri* at both the adult and pupal stages was consistently and significantly different from all of the other specimens, including wild-caught *An. sinensis*, colonized *An. sinensis* and '*An. lesteri*' identified by adult characteristics alone. The least amount of variation was seen within the colonized *An. sinensis*, as would be expected for colony-reared specimens. Images of the RAPD gels are included in this dissertation as Appendix C.

DISCUSSION

Wilkerson et al. (1995) noted the difficulty with determining whether morphological "variations, which are often slight between cryptic species, actually represent species differences or simply polymorphic characters within a species." To address such taxonomic difficulties Lounibos et al. (1998) suggested the use of a "multidisciplinary approach to mosquito systematics."

The current study revealed that over 90% of the specimens identified as *An. lesteri* by adult characteristics lacked pupal characteristics for that species. In addition, analysis with RAPD markers suggested that adults from the two putative species, *An. lesteri* and *An. sinensis*, were, in fact, not different from each other or from known *An. sinensis* specimens. The only exception to this conclusion was the one specimen that had both adult and pupal characteristics of *An. lesteri*. Finally, classification of the specimens according to the sequence of the COII gene was different from classification according to adult morphological

characteristics; no differences were noted between specimens in the DII gene sequence. Classification according to the sequence of the COII gene resulted in three genotypes, each of which contained specimens of both morphotypes, indicating that this gene was not useful in separating the two putative species. In addition, the number of mutations in the COII gene was relatively low compared to the *Culex pipiens/Cx.torrentium* study performed by Guillemaud et al. (1997). In that study, a total of 14 mutations was observed in a 657 base pair sequence; whereas, in our study, eight mutations were observed in a 547 base pair sequence.

Based on the above findings, we derived three conclusions about malaria vectors in the ROK. First, adult mosquito characteristics currently used to separate *An. sinensis* and *An. lesteri* are unreliable. Second, the use of adult morphology alone to separate malaria vectors leads to an overestimate of the relative abundance of *An. lesteri*. Third, most of the anophelines reared to the adult stage during this study were *Anopheles sinensis*.

This study suggests that about 92% of the specimens identified as *An. lesteri* in adult

collections in the ROK are misidentified. The consistency of findings between three different techniques -pupal morphology, gene sequencing and RAPD analysis- strongly indicates the need for further research into the systematics and identification of malaria vectors in the ROK. Moreover, this research suggests that pupal morphology may be a more reliable means of separating the species in question than adult morphology.

Finally, there is some doubt as to the real identity of *An. sinensis* in the northern part of its range (Bruce Harrison, personal communication). Some taxonomists believe that this mosquito is different from that of the type locale in southern China. Our study suggests a similarity with the putative *An. sinensis* from the northern part of the range, and this may not apply to specimens from the southern area.

Table 1. Primers used for comparison of wild-caught

mosquito specimens in the Kyungii Province, Republic of

Korea, 2000.

Designator	Sequence
A2	5'-TGCCGACGTG-3'
A3	5'-AGTCAGCCAC-3'
A10	5'-GTGATCGCAC-3'
A19	5'-CAAACGTCGG-3'
A20	5'-GTTGCGATCC-3'
B14	5'-TCCGCTCTGG-3'
C2	5'-GTGAGGCGTC-3'
C7	5'-GTCCCGACGA-3'
C10	5'-TGTCTGGGTG-3'
C13	5'-AAGCCTCGTC-3'
C18	5'-TGAGTGGGTG-3'
C20	5'-ACTTCGCCAC-3'

Table 2. Nei-Li distance matrix for two *Anopheles* species from the Republic of

Korea as determined by random amplification of polymorphic DNA.

Specimen	1	2	3	4	5	6	7	8	9
'*lesteri*'1[1]	-								
'*lesteri*'2[1]	.043	-							
'*lesteri*'3[1]	.072	.060	-						
'*lesteri*'4[1]	.041	.040	.047	-					
'*lesteri*'5[1]	.076	.041	.026	.064	-				
sinensis 1	.036	.043	.051	.031	.068	-			
sinensis 2	.048	.028	.055	.068	.048	.050	-		
sinensis 3	.052	.041	.060	.060	.052	.043	.047	-	
sinensis 4	.053	.052	.037	.040	.054	.055	.048	.052	-

[1] Specific identification based solely on adult characters; identification is questionable.

Table 2 (Con't)

Specimen	1	2	3	4	5	6	7	8	9
lesteri (p)[2]	.203	.225	.236	.206	.243	.213	.219	.275	.243
colonized 1	.046	.054	.053	.043	.046	.037	.051	.036	.056
colonized 2	.044	.052	.084	.061	.076	.054	.048	.042	.089
colonized 3	.047	.046	.088	.064	.068	.058	.043	.037	.092

[2] Specimen identified by both adult and pupal characters.

Table 2 (Con't)

Specimen	10^2	11	12	13
lesteri (p)	-			
colonized 1^3	.280	-		
colonized 2^3	.291	.021	-	
colonized 3^3	.297	.025	.003	-

[2] Specimen identified by both adult and pupal characters.

[3] Specimen from colony of known An. *sinensis* maintained in Shanghai, China.

Table 3. Median Nei-Li distance as determined by RAPD-PCR analysis between putative *Anopheles* species from Kyungii Province, Republic of Korea.

Comparison[1]	Median distance
An.'lesteri'[2]\colonized An. sinensis	0.291 A
An. 'lesteri'[2] \An. 'lesteri'[3]	0.230 A
An. 'lesteri'[2]\wild-caught An. sinensis	0.231 A
Wild-caught An.sinensis\colonized An.sinensis	0.052 B
An 'lesteri'[3]\colonized An. sinensis	0.053 B
Within group of wild-caught An. sinensis	0.049 B
An. 'lesteri'[3]\wild-caught An. sinensis	0.052 B
Within group of 'An. lesteri'[3]	0.045 B
Within group of colonized An. sinensis	0.025 C

[1] Medians followed by the same letter are not significantly different; Kruskal-Wallis, p > 0.05).

[2] Specimen identified by both adult and pupal morphological characteristics.

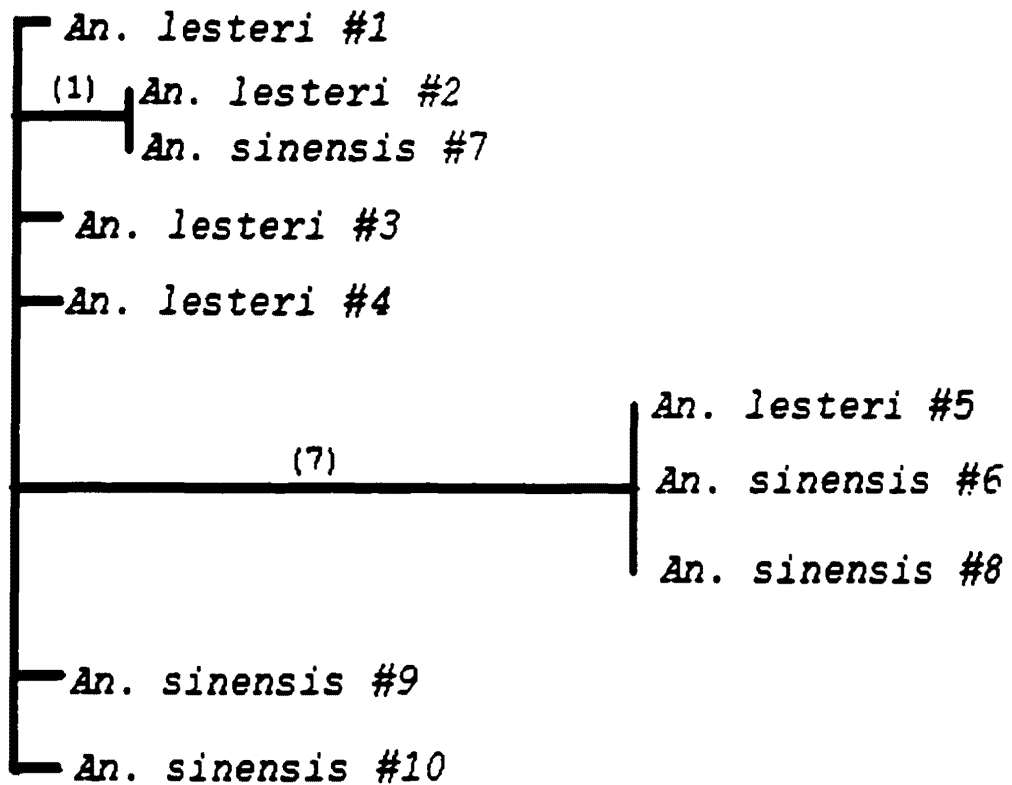[3] Specimens identified by adult morphological characteristics only.

Figure 1. Tree diagram of relationship between 10
Anopheles mosquitoes as determined by sequencing of the
COII gene. Numbers in parenthesis indicate the number of
mutations separating that branch of the tree from the next
nearest branch.

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FOOTNOTES

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CHAPTER 4

IKONOS® AND LANDSAT ESTIMATES OF MOSQUITO HABITAT IN KYUNGGI PROVINCE OF THE REPUBLIC OF KOREA

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For: Photogrammetric Engineering and Remote Sensing

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IKONOS® AND LANDSAT ESTIMATES OF MOSQUITO HABITAT IN KYUNGGI PROVINCE OF THE REPUBLIC OF KOREA

By

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IKONOS AND LANDSAT ESTIMATES OF MOSQUITO HABITAT IN KYUNGGI PROVINCE OF THE REPUBLIC OF SOUTH KOREA

Quantification of *Anopheles* larval habitat for malaria control in the Republic of Korea can be improved using IKONOS imagery to verify the Landsat classification results.

IKONOS AND LANDSAT ESTIMATES OF MOSQUITO HABITAT IN
HYUNGGI PROVINCE OF THE REPUBLIC OF KOREA

By

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GORDON AND RICHARD G. ANDRE

ABSTRACT

Malaria re-emerged in the Republic of Korea
(ROK) in 1993 and the number of cases increased
geometrically until 2000, when transmission seemed to
stabilize. U.S. soldiers in the affected area near
the Demilitarized Zone use chemoprophylactic drugs to
prevent malaria, but control of *Anopheles* mosquito
larvae also can be used to reduce transmission risk.
In order to compare the cost of larviciding versus
chemoprophylaxis, accurate estimates of the size of
anopheline mosquito larval habitats are necessary.

To estimate the size of larval habitats near Camp
Greaves in the ROK, an Ikonos and a Landsat image were
classified using a parellelpiped classification algorithm.
Ponds and rice fields were accurately classified on the

Ikonos image. Ponds could not be classified separately on the Landsat image because the ponds were too small to select training sites. However, some larger ponds were identified on the Landsat image as part of the river class. Ponds covered a very small area compared to rice fields but had high larval densities, so locating ponds could be very important for a mosquito control program. Ditches could not be accurately classified on the Ikonos image possibly due to the trees and other vegetation that tend to grow next to the ditches. Comparing the classifications on a pixel-by-pixel basis, the agreement between the two classifications was 79%. Part of the disagreement was due to the difference in resolution between the two images; Landsat classified the small roads and dikes between rice fields as rice paddy, but Ikonos could distinguish between the two. In spite of local differences in the two classifications, overall classifications produced similar land cover estimates.

BACKGROUND

After being absent from the Republic of Korea (ROK) since the 1970's, malaria (*Plasmodium vivax*) re-emerged with the occurrence of 2 cases in 1993. The number of cases has grown almost every year since, resulting in 1,642 cases in 1997 (Feighner et al. 1998). More than 4,000 cases of malaria have been diagnosed since the disease re-emerged, with more than 2% of the cases occurring in U.S. military personnel stationed in the ROK (Preventive Services Directorate, 18th Medical Command, Seoul, ROK; personal communication). The focus of the disease has been just south of the Demilitarized Zone (DMZ) between the Republic of Korea (ROK) and the People's Democratic Republic of Korea (PDRK). The primary vector of malaria in South Korea is *Anopheles sinensis* Wiedemann, a species that is highly associated with the rice paddy environment (Tanaka et al. 1979).

Since 1999, US Army personnel stationed near the Demilitarized Zone(DMZ) have used chemoprophylactic drugs and other preventive measures such as insecticide-impregnated bed nets and uniforms, as well as topical repellants. The use of larvicides in the rice paddies and ponds surrounding the

military bases is another option that has been suggested (Strickman et al. 1999). This study is part of a larger effort to determine the utility and cost of various methods to reduce the risk of malaria for U.S. military personnel. The purpose of this study was to determine whether an accurate estimate of the area covered by mosquito habitat could be obtained using Landsat and/or Ikonos data for the Korean test site. The area estimates of mosquito habitat can then be used to estimate the cost of larviciding near U.S. military bases.

The use of remote sensing to detect mosquito breeding habitats has been shown to be possible by several authors. Remote sensing has been used to predict which California rice fields will have the highest production of *An. freeborni* larvae nearly two months before the peak larval density occurs (Roberts and Rodriguez, 1994; Wood et al., 1991). Beck and others (1994) used images from Landsat Thematic Mapper (TM) to estimate the risk of malaria in forty villages in Chiapas, Mexico, based on the presence of two environmental factors: transitional swamp and unimproved pasture. Rejmankova and others (1998) used classified multispectral SPOT data to identify marshes containing vegetation favorable for mosquito habitat in Belize. The current study utilized Landsat and IKONOS

images to quantify the size of larval habitats within the vector's flight range around two U.S. Army bases in the ROK.

Ikonos imagery is a commercial product acquired by the Ikonos satellite and sold by Space Imaging, Inc. Characteristics of the Ikonos and Landsat bands are listed in Table 1. The obvious difference is in the resolution of the two sensors: 15 and 30 meters for the Landsat panchromatic and multispectral bands, respectively, versus 1 and 4 meters for the Ikonos panchromatic and multispectral bands. One of the questions that this study was designed to answer is whether the high resolution imagery is more effective in delineating certain small mosquito habitats, and if so, how this affects the overall estimate of the extent of the habitat in the low resolution image.

METHODS

Field work

Field work for this project was performed from June through September, 2000, and concentrated on two military bases near the DMZ: Camp Greaves and Camp Casey. Camp Greaves is located in a rural area just south of the DMZ. Camp Casey is approximately 56 KM east of Camp Greaves and is in a more populated area with less agriculture.

Standard larval survey techniques using a plastic dipper in all types of standing fresh water were carried out at both sites. Seven types of larval habitats were identified and sampled: 1) rice fields, 2) streamside pools 3) irrigation ponds, 4) irrigation ditches, 5) drainage ditches, 6) swamps, and 7) rivers. Only two small swamps occurred in the study area and were determined not to be important larval habitats. The river at Camp Greaves could not be sampled due to military security measures, but a nearby upriver site was sampled with negative results.

Each field site was located by using a Garmin III Global Positioning System (GPS) unit. Since GPS readings could not be taken in the center of the rice fields due to potential crop damage, four readings were taken at the

157

corners of the field and then the points were averaged to

obtain an estimate of the center of the field.

GPS points were plotted on topographic maps in the

field using ArcView. After the completion of the field

work, points also were displayed on the Ikonos image.

Almost all of the 93 points collected were accurate

with generally no more than about five meters of

offset. A few points had a greater locational error,

possibly due to electromagnetic interference from

nearby towers and transmission lines. Offset points

were manually corrected using the topographic maps or

IKONOS image as a reference.

Because of the interest in estimating larval

habitat for areas that would affect the camps, buffer zones

were created around the two camps using Arc/Info software.

The boundary of Camp Casey was digitized and a 1-kilometer

buffer zone was placed around the camp based on the

approximate 1-kilometer flight range of the mosquito

(Strickman, 1999). Due to the size and shape of Camp

Greaves, a 2-kilometer radius circle was created around a

point marking the approximate center of Camp Greaves,

resulting in an approximate 1-kilometer buffer zone around

the perimeter of the camp. Figure 1 shows the larval

sampling sites and the 1-kilometer buffer zone around Camp Greaves.

Analysis of Imagery

Two images were used for this study, a Landsat image acquired on April 29, 2000, and an Ikonos image acquired on August 2, 2000. The Landsat image covers both the Camp Greaves and the Camp Casey site. The Ikonos image only covers the Camp Greaves site. The images were georeferenced to a UTM projection and a WGS-84 datum. GPS points taken in the field plotted accurately on the Ikonos image. A visual comparison of the images with each other and with georeferenced topographic maps showed that the geometric correction of the images was good.

PCI's remote sensing software was used to perform supervised classifications on the Ikonos and Landsat images. Training sites were selected at locations where researchers had sampled standing water for anopheline larvae. Various classification algorithms were tried including minimum distance, maximum likelihood and parallelepiped programs. The parallelepiped algorithm with maximum likelihood as a tie breaker, appeared to be the best classification of the images based on a

visual comparison with a plot of the sampling sites on the Ikonos image. For the IKONOS image, training sites were collected for the river, ponds, ditches, and rice fields. Because of the lower resolution, training sites on the Landsat image included only rice fields and the river; ponds and ditches were too small to be seen. The river, which could not be sampled in the Camp Greaves area due to security measures, was considered to be non-habitat based on sampling results at sites upriver. Other areas classified as urban and forest were grouped together as a single, non-habitat class for the purpose of estimating area.

PCI's MODEL program was used to generate reports on the area covered by each class in the classified image. The MODEL program also was used to compare the Landsat and Ikonos classifications on a pixel-by-pixel basis and create a new image that depicted matching and non-matching pixels.

RESULTS

Figures 2 and 3 show the result of the classifications of the Landsat and Ikonos images. Rivers, ponds and rice fields were successfully classified on the Ikonos imagery. Ditches could not be successfully classified on the Ikonos imagery, possibly due to the trees, shrubs and other vegetation that grow along the ditches, which make them spectrally similar to other land, cover classes. On the Landsat imagery, rivers and rice fields could be classified, but ponds and ditches were too small and could not be used for "collecting training sites". (This term refers to the selection of pixels from a known geographic location for extrapolation of larval sampling results to other pixels with similar reflections). A visual comparison of the Landsat and Ikonos classifications, shown in Figures 2 and 3, shows that the two are quite similar in the classification of the river. In Figure 3, on the Landsat classification, large areas of rice fields were classified fairly accurately. However, small rice fields, as seen on the Ikonos image in Figure 3 in the NW corner of the image, were not accurately classified on the Landsat classification. A comparison of

the Landsat and Ikonos classification was done using the PCI MODEL program to compare the classification on a pixel-by-pixel basis. White pixels in Figure 4 represent the pixels that were classified the same on the 2 images; black pixels were classified differently. A report generated by PCI's MODEL program calculated that 79.4% of the image was classified the same on Landsat and IKONOS.

More modeling was done to explore the differences in the two classifications. In Figure 5, black pixels represent areas that were classified as habitat on Landsat but as non-habitat on Ikonos. One reason for the difference is that Landsat classified the small roads and large dikes separating the rice fields as rice paddies because of lower resolution; whereas, Ikonos classified them as non-habitat. There also may be some mixed pixels on the Landsat, which were classified as rice field but were separated into rice and non-habitat on the Ikonos image.

In Figure 6, black pixels represent areas that were classified as habitat on Ikonos but as non-habitat on Landsat. Larger patches of black on this image represent fields that were inaccurately classified as non-habitat on the Landsat. A comparison with the Landsat image shows that some of these fields are obviously spectrally different from the other rice fields and may represent a

different stage of rice cultivation (non-flooded versus flooded fields). Some of the small rice fields that were classified as non-habitat also are apparent on Figure 6. A comparison of Figure 6 with Figure 2 shows that a scattering of pixels from forested areas were misclassified as rice fields on the Ikonos image.

Area estimates for the buffer zone around Camp Greaves are shown in Table 2. Although the difference in the classification of Landsat and Ikonos images was approximately 20%, the area estimates were very close.

CONCLUSIONS

We have found that similar land cover area estimates of mosquito larval habitat can be obtained from IKONOS and Landsat imagery. The use of Ikonos has the advantage of being able to portray and classify small land cover features such as ponds and rice fields less than 30 by 30 meters in size. Although ponds represent a relatively small portion of the total habitat area, they are an important breeding habitat for mosquitoes because they contain higher larval densities than the rice fields late in the growing season (Claborn et al., in review). Given that Landsat imagery is cheaper to acquire and easier to process (fewer scenes and pixels to cover the same area), but Ikonos provides more detail, which imagery type should be used? For areas where small features represent the majority of the habitat, the extra expense of acquiring the Ikonos imagery may be worthwhile. Also, Ikonos imagery would be extremely useful in planning sampling collections and larviciding tasks. However, for overall cost estimates of larviciding and other types of program planning which need only rough estimates of the major habitat areas

defined, Landsat would be adequate. The best use of these types of data might be to use the Landsat imagery in conjunction with Ikonos imagery. Ikonos images could be used to verify the land cover estimates in several places on the Landsat image to help assure that the estimates are accurate.

These habitat land cover estimates are used to estimate the cost of larviciding around the camps as a control method (Claborn et al., in review). Larviciding cost estimates then will be compared to the cost of chemoprophylaxis for U.S. personnel stationed at Camp Greaves and Camp Casey. Depending on the results, this approach may prove useful for cost estimates of larviciding for other military camps in the ROK.

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Table 1. Band characteristics of Ikonos and Landsat images.

Image	Band		Wavelength(um)	Resolution(m)
IKONOS	1	Blue	0.45 - 0.52	4
	2	Green	0.52 - 0.60	4
	3	Red	0.63 - 0.69	4
	4	Near-Infrared	0.76 - 0.90	4
		Panchromatic	0.45 - 0.90	1
Landsat	1	Blue-green	0.45 - 0.52	30
	2	Green	0.53 - 0.61	30
	3	Red	0.63 - 0.69	30
	4	Near-Infrared	0.75 - 0.90	30
	5	Near-Infrared	1.55 - 1.75	30
	6	Thermal	10.4 - 12.5	60
	7	Near-Infrared	2.09 - 2.35	30
	8	Panchromatic	0.52 - 0.90	15

Table 2. Comparison of land cover area estimates (m^2) for Camp Greaves, Republic of Korea

Class	Image	
	Ikonos	Landsat
Rice paddy	4,198,151	4,304,250
Ponds	48,709	---
River	1,465,431	1,604,925
Non-habitat (other than river)	6,789,022	6,502,500

Figure 1. Ikonos image with 1-km buffer zone around Camp
Greaves. Larval sampling sites are noted with white dots.
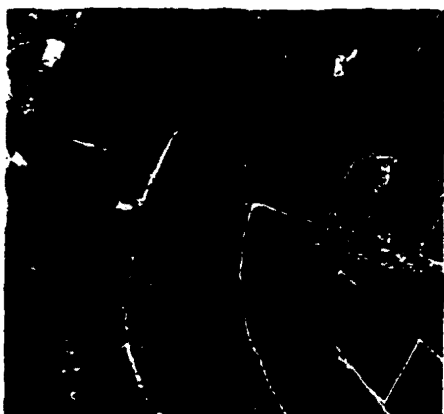
Ikonos image

Landsat image
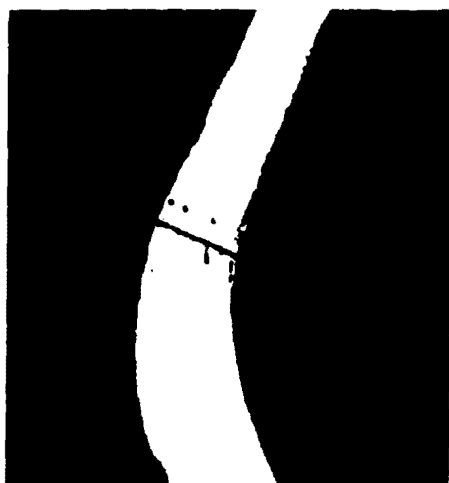
Ikonos classification

Landsat classification

Figure 2. Comparison of Ikonos false color composite and classification with Landsat. On the classification images, the river is shown in yellow, rice fields in green, and ponds in white.

Ikonos Image   Landsat Image

Ikonos Classification   Landsat classification

Figure 3. An enlarged subset of the images from figure 2
comparing an Ikonos false color composite and
classification with Landsat. On the classification images,
the river is shown in yellow, rice fields in green and
ponds in red.

Figure 4. Image showing agreement of pixels in the
classification of Landsat and Ikonos images. Black
pixels (20.6% of image) were classified differently in
the two images. White pixels were assigned to the same
class.

Figure 5. Image showing pixels (in black) that were classified as habitat on Landsat but as non-habitat on Ikonos.

Figure 6. Image showing pixels (in black) that were classified as habitat on Ikonos but as non-habitat on Landsat.

176

CHAPTER 5

REMOTE SENSING AND GEOGRAPHIC INFORMATION SYSTEMS AS

DECISION-SUPPORT TOOLS FOR THE CONTROL OF MALARIA

(*PLASMODIUM VIVAX*) IN KYUNGGI PROVINCE, REPUBLIC OF KOREA

For: American Journal of Tropical Medicine and Hygiene

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REMOTE SENSING AND GEOGRAPHIC INFORMATION SYSTEMS AS
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REMOTE SENSING AND GEOGRAPHIC INFORMATION SYSTEMS AS DECISION-SUPPORT TOOLS FOR THE CONTROL OF MALARIA (*PLASMODIUM VIVAX*) IN KYUNGGI PROVINCE, REPUBLIC OF KOREA

by

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ABSTRACT A cost-comparison of two methods for the control of malaria in the Republic of Korea was performed. The cost of larviciding with methoprene granules was estimated at $93.48/hectare. The annual cost of providing chemoprophylaxis was estimated at $37.53/person. Remote sensing and geographic information systems (GIS) were used to obtain estimates of the size of vector larval habitats around two U.S. Army camps, allowing an estimate of the cost of larviciding around each of the camps. This estimate was compared to the cost of providing chloroquine and primaquine chemoprophylaxis for the camp populations. The use of remote sensing and GIS allowed a feasibility analysis for larval control measures within a 1-km radius of each camp based on a cost comparison of malaria reduction strategies. Costs on each of the camps differed by the size of the larval habitats and the size of the at-risk population. On one camp, a single larvicidal application was more expensive than chemoprophylaxis;

whereas on the other camp, 29 larvicidal applications could be made for the cost of providing chemoprophylaxis. These tools allow extrapolation of larval surveillance data to a regional scale while simultaneously providing site specific cost analysis, thus reducing the cost and labor associated with vector surveillance over large areas.

INTRODUCTION Malaria (*Plasmodium vivax*) is a re-emerging disease in the Republic of Korea (ROK).[1] After an absence of more than ten years, two autochthonous cases were diagnosed in 1993. Malaria increased exponentially until 1998, when the number of reported cases stabilized. More than 4,000 cases were reported through CY 2000. Among all the cases, approximately 2% occurred in American military personnel stationed in the ROK (Preventive Services Directorate, 18th Medical Command, Seoul, ROK; unpublished data). The strain of *P. vivax* transmitted in the ROK is well adapted to a temperate climate and demonstrates both short and long incubation periods. Long incubation periods of greater than six months increase the risk of importing malaria to the United States, with potential for establishing autochthonous transmission. This increased risk is due to the delayed onset of symptoms, which may not occur until the soldier has returned to the U.S. or even left the U.S. Army. The control of malaria in military personnel is very important, not only to reduce the risk of importing and establishing transmission in the U.S., but also to maintain military readiness.[2]

The primary malaria vector in the ROK is *Anopheles sinensis* Wiedemann, though secondary vectors may include

the less commonly collected *An. lesteri* Baisas and Hu and *An. yatsushiroensis* Miyazaki. A strong association of these species with rice culture has been noted many times.[3,4,5] Also, *An. sinensis* larvae have been collected from the irrigation ditches and ponds contiguous to the rice paddies. Ponds may support the highest densities of malaria vectors late in the growing season (Claborn, unpublished data). The high densities of malaria vectors in irrigation pools and the large number of wetland rice paddies make these two habitats the primary larval habitats of malaria vectors in Kyunggi Province, ROK. Any larval control efforts as part of an overall malaria control program would have to significantly reduce the larval populations in the rice culture environment.

Larviciding is only one of several malaria control methods with potential for use in the ROK. Techniques such as early case detection and treatment, personal protective measures (repellents, bed nets, etc.) and mosquito adulticiding are all used to some degree by the U.S. Army as part of their malaria prevention program. Chemoprophylaxis and larviciding have both been considered for the protection of U.S. Army personnel in the ROK.

The degree to which larviciding and chemoprophylaxis are appropriate for the protection of

American military personnel operating in malarious regions is not well known. Variables that impact the decision-making process in selection of malaria control measures include the relative efficacy of the various methods, unintended effects, and the implementation costs, as well as cultural, historical and political factors. Cost is often considered to be one of the more important variables, yet few studies have been conducted comparing the costs of disparate control measures like chemoprophylaxis and larviciding. In contrast, many cost comparisons and cost-effectiveness studies have been performed recently on the applicability of insecticide-treated bed nets in malaria control programs.[6,7,8] One study compared the costs of several disparate control methods in Africa, including insecticide treated bed nets, residual sprays for adult mosquito control, chemoprophylaxis for children, intermittent treatment of pregnant women and improvement of case management.[9] Cost comparisons for various malaria control methods in Africa may not be applicable to proposed programs in the ROK due to the presence of multispecific malaria in the former, as well as significant differences in mosquito bionomics and behavior. Furthermore, there have been no published cost-comparisons of the two control methods of interest to the U.S. Army in

the ROK: larviciding and chemoprophylaxis. Part of the reason for the lack of such studies is the large amount of time and personnel required to obtain the data required to quantify the associated costs, especially for larviciding programs. Remote sensing in the form of satellite images provides a method for obtaining the requisite data quickly, cheaply and reliably.

One of the primary variables in determining larviciding costs is the size of the area to be treated; this can be measured accurately with a geographic information system (GIS). A GIS is a computer software package that stores and analyzes geographical information. Remote sensing and GIS are powerful tools that associate vector occurrence and abundance within a geographical perspective. The information can then be used to predict vector abundance, identify habitats that are likely to harbor vector populations, and analyze risk of disease transmission. This technology has been utilized in the study of numerous vector-borne and zoonotic diseases including African trypanosomiasis[10,] schistosomiasis[11], Lyme disease[12] and Rift Valley fever.[13] Geographic information systems also have been utilized to study malaria vectors in Central and South America.[14,15] Remotely sensed images and GIS also can be used to determine the size and location of

vector larval habitats, thus allowing an estimate of the cost of larviciding efforts.

This paper reports the use of GIS and remote sensing to estimate the cost of larviciding for a cost-comparison with chemoprophylactic prevention of malaria in the ROK.

MATERIALS AND METHODS Standard larval sampling techniques were used to sample habitats around two U.S. Army camps (Camp Greaves and Camp Casey) in Kyunggi Province, ROK, from June through September, 2000. Initially, 60 sites were sampled monthly, but other sites were added throughout the study period for a total of 93 sites: 50 rice paddies, 13 stream pools, 12 irrigation ditches, 11 drainage ditches, 5 irrigation pools, and 2 swamps. Larvae were identified to genus at the time of sampling, and a sample of the *Anopheles* was reared to the adult stage for specific identification and for maintenance of voucher specimens. Specific identifications and inter-habitat comparisons will be reported elsewhere. The location of each site was plotted with a Garmin III® global positioning unit.

A Landsat image acquired on 29 April, 2000, was used for GIS analysis. The image was classified as non-habitat, river, and rice paddy using the EASI ® software package. These areas were classified based on "ground-truthed"

knowledge of the habitat at sampled sites. A buffer was constructed around each camp based on the flight range of *An. sinensis,* reported to be about 1 km.[3] Due to the size and shape of Camp Greaves, the buffer was a circle with a 2-km radius centered at the camp's center. The shape of Camp Casey did not allow such a simple buffer, so the shape of the base was digitally placed in the image and a 1-km buffer constructed from the perimeter of the camp. All habitats inside the buffer were quantified, including those on the camps. The general rice paddy habitat that includes ditches, ponds and paddies, was considered to be vector habitat; all others, including the river at Camp Greaves, were considered to be non-habitat.

Calculation of insecticide application costs

A cost analysis of rice farming in Mississippi identified twelve variable costs.[16] Although there are numerous differences between rice farming in Mississippi and the ROK, four of the variables were obviously applicable to larviciding: repairs and maintenance, fuel, operator labor, and overhead labor. In 1996 and on a per acre basis, the costs of these variables for Mississippi rice farming were estimated

at $29.69, $28.11, $16.58 and $10.00, respectively. However, these estimates include costs for many different operations over the length of the growing season, including planting, harvesting and the application of pesticides, fertilizers and lime. In addition, fuel costs in the ROK are greater and labor costs are significantly lower than those in the U.S. The agricultural wage rate in the ROK in 1996 was estimated to be $33.30 U.S. dollars/day ($4.16/hour for an eight-hour day).[17]

The cost of applying larvicides to mosquito habitats in the ROK was estimated using six assumptions.

(1) A granular formulation of methoprene, an insect growth regulator) is used at a rate of 5.6 kg/ha and a mean cost of $73.97/hectare (ha).

(2) A trailer-mounted power applicator is utilized with the capability of applying 2.27 kg/minute.

(3) A 3-person team working 8-hours each day can

cover a total of 97 hectares. (This assumes that half of the workday is taken up in transportation, machine preparation and loading of the insecticide.)

(4)   Each person is paid $4.50/hour.

(5)   Existing equipment is used.

(6)   Contractors are allowed a 10% mark-up.

With these assumptions, variable costs per hectare were estimated at $1.12 (labor), $73.98 (insecticide), $4.94 (fuel) and $4.94 (repair and maintenance). Fuel assumptions were based on an estimated cost of $2.00/acre, then translated to hectares. In the Mississippi study, fuel and maintenance costs were equivalent, so they they were assumed to be equivalent for this study as well. Total internal cost, therefore, was estimated at $84.98/ha; contracted cost would be $93.48/ha.

Calculation of chemoprophylaxis costs

The cost of chemoprophylaxis is a function of
the number of people needing protection, the cost of
the prescription, the length of time drugs are
required, and packaging costs. Other costs that might
be considered include the cost of treating side-
effects and failures, but these variables are beyond
the scope of this study. Given the small number of
cases in American personnel and the low rate of side
effects from chloroquine, the costs attributed to
side-effects and treatment failures would be minimal.
However, the cost of treating side effects from
primaquine could be significant. To minimize this
cost, testing for glucose-6-phosphate dehydrogenase
(G-6-PD) deficiency is required for U.S. soldiers
deployed to Korea before they are issued primaquine.
Since U.S. Army soldiers are not routinely tested for
G-6-PD deficiency in basic training, the cost of this
test was added to the overall cost of
chemoprophylaxis. Assuming drug costs of $0.92 and
$0.42 per tablet for chloroquine and primaquine,
respectively, a 19-week period of chemoprophylaxis
including four weeks of terminal prophylaxis, a

medication packaging cost of $0.39 per person, and a single G-6-PD test at a cost of $6.00, the initial cost per person of chemoprophylaxis was calculated to be $31.20. This estimate assumes that any soldier who rotates out of the area is immediately replaced by another who immediately starts chemoprophylaxis. However, a high percentage of personnel rotations in the middle of the malaria season results in a greater number of terminal prophylactic regimens. Assuming that half of the soldiers rotate during the malaria season, that would add the cost of another half-regimen of primaquine, half the cost of one G-6PD test, and two weeks of chloroquine to the per person cost for a total of $37.53. Chemoprophylaxis prior to arrival in high-risk areas of the ROK is not prescribed, which is actually contrary to standard recommendations.

RESULTS Three *Anopheles* species were collected as larvae and reared to the adult stage during the study- *An. sinensis* Wiedemann, *An. lesteri* Baisas and Hu, and *An. yatsushiroensis*. Because all of these species are potential malaria vectors, any area with *Anopheles* larvae was considered a vector habitat. Nearly all

stagnant or slowly moving fresh water sites in the study area were potential vector habitats (Claborn, unpublished data), so all rice paddies and associated ditches and ponds were classified as vector habitat. Initial classifications were performed on a 1-meter resolution Ikonos image (Space Imaging, Inc.), then the same areas were classified on a Landsat image. There was a 79.4% agreement in areas classified as vector habitat by the two systems. Although the IKONOS image possesses greater resolution and thus better accuracy, it is much more expensive and was available for only a limited area due to persistent clould cover. Therefore, the Landsat classification was used for analysis. Figure 1 is a Landsat image that is classified for larval habitat status in a 1-km buffer around Camp Greaves. Figure 2 is a similar image for Camp Casey. The larger amount of habitat in the Camp Greaves image is due primarily to the area's expansive fertile valley with associated rice farming. As each pixel in the images represents 900 m^2, the size of each habitat classification can be determined easily. These estimates are reported in Table 1 for both camps. The size of the rice paddy habitats was 430 ha for Camp Greaves and 123 ha for Camp Casey. At

an in-house cost of $84.98/ha, the cost of larviciding

rice paddies around Camp Greaves would be $36,575;

whereas, the cost for Camp Casey would be $10,410.

Contracted costs would be $40,232.50 and $11,451,

respectively. These estimates are for one treatment

only. We assumed that three treatments would be

required during the entire rice-growing season.

The active duty population at Camp Casey is

approximately 6,300 soldiers; another 2,500 civilians also

live and work there. Camp Greaves is much smaller with a

total of approximately 600 military and 160 civilians.

Assuming a per capita cost for chemoprophylaxis of $37.53,

the costs of treatment for all personnel at Camp Greaves

would be $28,522.80 and at Camp Casey would be $330,264.

Thus, a one-time contracted larvicide application at Camp

Greaves would be more expensive than chemoprophylaxis. The

cost of three applications would be 4 times greater than

the cost of chemoprophylaxis. In contrast, more than 28

larvicide applications could be applied to the habitats at

Camp Casey for the cost of providing chemoprophylaxis to

the resident population. If chemoprophylaxis were only

provided to active duty military personnel at Camp Casey,

the cost would still exceed that of larviciding by 21

times.

DISCUSSION This study reports the use of remote sensing and GIS as decision-support tools in the selection of appropriate measures for malaria control in the ROK. Although these strategies have broad applications in the control of vector-borne disease, environmental, epidemiological and socio-political characteristics in the ROK must be carefully considered for an effective control program. For example, malaria transmission in the ROK is not typical of tropical malaria in that a proportion of the infected human population does not demonstrate symptoms until 6-9 months after infection.[2] The malaria transmission season is limited by the cold winters that terminate vector activity. Since malaria infected mosquitoes do not overwinter, malaria begins anew each spring through non-infected mosquitoes feeding on *P. vivax* gametocyte carriers and/or patients becoming parasitemic from infections acquired 6-9 months earlier. Chemoprophylaxis is required for only a relatively brief period of time every year. Seasonal transmission of malaria and variable incubation periods have direct impact on the costs and applicability of chemoprophylaxis and larviciding, as well as other malaria control control methods.

In this study, the larval habitats around military facilities were surveyed and analyzed to provide a basis for cost estimates of larviciding. However, the same techniques can be used for civilian population centers. This remote sensing technique allows the user to "extrapolate measurements made at a local level to a regional or global scale".[18] However, remote sensing does not replace field surveys of larval habitats; it simply reduces the amount of field work required. Based on limited mosquito surveillance conducted around population centers, estimates can be made of the dimensions and area of larval habitats around other villages, cities or military bases. This information, correlated with human population size, variable costs of application and treatment, biology of the vector, disease epidemiology, and socio-political characteristics, allows for informed cost comparisons of different malaria control methods. This study demonstrates that variations in at-risk populations and larval habitat sizes can result in significant differences in the cost estimates for different malaria control methods. Although this conclusion seems obvious, available data may not permit reliable cost estimates. Remotely sensed images allow the identification and quantification of potential mosquito habitats that may

easily be missed during field surveys. This capability is especially useful when the survey is conducted by persons who are unfamiliar with the geographic and socio-political environment (i.e., military personnel or other specialists working in a foreign country).

The socio-political environment, including both civilian and military populations, is an area of primary concern when developing malaria control strategies and may affect the feasibility of implementing methodologies. In the ROK, rice growing areas are privately owned, and any mosquito control efforts require approval of the Korean National Institute of Health, local public health officials, the Ministry of Defense and the landowner. Less than complete compliance with larviciding efforts may negate costs and manpower expenditures for malaria control. U.S. soldiers often train at remote areas that are joint ROK-US training ranges and are suspected high-risk malaria sites. Mosquito surveillance on these sites requires the approval of and coordination with the Korean Ministry of Defense. Application methods with larvicides are also limited by location because the ROK and U.S. seek to avoid provocation by prohibiting aerial application near the "no-fly" zone of the DMZ.

In addition to potential socio-political barriers, cost estimates are limited by several other factors. Variable costs for drugs, insecticides, fuel, and labor can change over time and necessitate a recalculation of total costs. Also, if existing insecticide-dispersal equipment is not available, the cost of new equipment and depreciation of that equipment must be included in the cost of larviciding. Utilization of another technique, such as aerial application of the insecticide, would also require further analysis. Similarly, the cost of instituting a drug dispensing system must be calculated if one does not already exist. In the current study, the existing military medical care system provides a mechanism to dispense chemoprophylaxis without added program costs.

Another limitation is that all potential habitats are included in the larvicidal treatment estimate. The use of surveillance, either direct or remote, could reduce the size of the treatment area and thus the expense of larviciding by treating only those sites that support detectable vector populations. Environmental parameters could be used as surrogates of high larval density and this knowledge could result in reduced cost of a larval surveillance program. Such parameters might include residues of agricultural chemicals in the floodwaters,

salinity, or length of time since the last flooding. Further work in this area is required.

Application of remote-sensing techniques to support decision-making on vector control strategies must be accompanied by caveats as well. There is some danger that assumptions about the biology of the vector and the surrounding environment may be applied in areas for which those assumptions are not valid. For example, this study was conducted in areas near the DMZ between the ROK and the Peoples Democratic Republic of Korea (PDRK or North Korea). There are large differences in the ecologies of the areas on either side of the DMZ that are readily apparent on the LANDSAT images. Environmental dissimilarities between the ROK and the PDRK, even though field data were collected only a few hundred meters from the border, may result in misapplication of the data that was collected only in the ROK. Thus, care must be taken to avoid extrapolating to too large of an area or in areas where the ecology is significantly different from "ground-truthed" areas.

This study is also limited by the incomplete knowledge of control method efficacy, especially for larviciding with methoprene. Although Weathersbee and Meisch (1991) achieved long-term control of mosquitoes with methoprene in Arkansas ricefields, their work dealt with

non-anopheles mosquitoes and the application of their data to malaria control is tenuous. Other studies, however, suggest a high level of methoprene efficacy against *Anopheles*. Laboratory studies by Ritchie et al.[19] revealed a wide range of minimum lethal doses for several mosquito species, from 0.5 ppb for *Ae. vigilax* to 40 ppb for *Cx. sitiens*. *Anopheles farauti* was highly susceptible and all mosquitoes were killed at a concentration of 4 ppb. Another laboratory study of methoprene showed that *An. stephensi* larvae did not suffer significant mortality but that 75% of the pupae died within 2-3 hours and that adult emergence from the pupae was also reduced.[20] Few field studies of methoprene against malaria vectors have been completed, but Kanda et al.[21] using another similar IGR (pyripoxifon), achieved good control of *An. minimus* and *An. maculatus* in slowly-moving streams. Moreover, no incident malaria cases were noted in treated areas at eight weeks post treatment, even though malaria transmission continued in the control areas. The lack of strong evidence for control of malaria with methoprene larvicide, therefore, necessitates a field efficacy test in the ROK prior to the implementation of any larvicidal program.

Another caveat is that areas will inevitably be misclassified by the software and the area of a habitat

will be over- or underestimated. In the LANDSAT image of Camp Casey, lawns that surround military barracks were classified as rice paddies and were counted as vector habitats. Although this misclassification resulted in minimal errors (less than 1% of the total pixels), it illustrates the requirement for extensive knowledge of the general area under study and the continued need for "ground-truthing".

In summary, the use of remote sensing and GIS to identify and estimate the size of larval habitats is a valuable decision-support tool for cost comparisons of malaria control strategies in specific geographic regions. This technology, in combination with epidemiologic data, as well as sociopolitical and environmental considerations, will result in informed decision-making for the control of malaria.

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Table 1. Area (m^2) of habitats within flight range of *Anopheles sinensis* around two U.S. Army camps in the Republic of Korea as depicted in LANDSAT images classified as to habitat. (Image was acquired on 29 April, 2000.)

Habitat	Camp Greaves	Camp Casey
Vector habitat[1]	4,304,250	1,224,900
River	1,604,925	---
Non-habitat	6,502,500	19,950,525

[1] Vector habitat includes rice paddies and associated irrigation ditches, ponds and streamside pools.

Figure 1. Classified Landsat image within 1-km buffer around Camp Greaves, ROK. Green represents *Anopheles* larval habitat, black is non-habitat and yellow is the Imjim River.

8 km

Figure 2. LANDSAT image of 1-km buffer around Camp Casey complex, ROK. Gray represents *Anopheles* larval habitat; black represents non-habitat.(Image acquired on 29 April, 2000).

CHAPTER 6

CONCLUSIONS

CONCLUSIONS

Initial field work in this study provided two summary assessments as foundations for all subsequent conclusions.

(1)   The vast majority of *Anopheles* in the rice field habitats of northern Kyunggi province are *An. sinensis*, the primary malaria vector in the ROK.

(2)   The high level of oviposition site adaptability exhibited by *An. sinensis* results in the suitability of most stagnant or slowly-moving freshwater as larval habitat for the vector.

The first conclusion allowed subsequent GIS analysis of targeted habitat size to be based on the occurrence of any *Anopheles* larva rather than a specific species. The second conclusion allowed the classification of specific, large, remotely-sensed areas as vector habitats. However, both of these conclusions come with caveats.

Although the majority of *Anopheles* reared from larvae for this study was *An. sinensis*, a concurrent

210

adult trapping study indicated a larger proportion of other species, specifically *An. lesteri* and *An. yatsushiroensis.* As a result of this dissertation, there is now considerable doubt as to the accuracy of *An. lesteri* identifications based solely on adult morphological characteristics. This work does not disprove the presence of this species in the ROK; it does, however, suggest that the abundance of *An. lesteri* is overestimated when identification is based solely on adult characteristics. Although not addressed in this study, similar taxonomic confusion also may exist with identification of *An. yatsushiroensis.* Therefore, further work on the systematics of this complex is very important. These identifications are critical given the uncertain vectorial status of *An. lesteri* and *An. yatsushiroensis* in the ROK. All *Anopheles* larvae reared during this study were assumed to be potential vectors based on the high proportion of *An. sinensis*.

The use of remote sensing for this study was intentionally limited (or targeted) in order to demonstrate the direct application of this technology for public health decision makers. The use of GIS and digital images to identify and quantify the size of

larval habitats has applications outside the narrow field of malaria control. For instance, public health officials might want to compare the long-term costs of vector control through larviciding with the cost of immunization for the control of Japanese encephalitis (JE) in rice growing areas of Asia. In many areas, the primary vector of JE, *Cu. tritaeniorynchus* Giles, is highly associated with rice paddies. Knowledge of the size of the larval habitats of this vector, coupled with the resulting cost of vector control, could be compared directly to the cost of immunizing a nearby population. In the case of JE, the $100.00/person cost of immunizing could very easily increase the attractiveness of larviciding, at least in the short term. When populations are highly mobile or transient (as with the military population), the long-term cost per person-year of protection would be extremely high for a JE immunization program. The cost of larviciding, of course, would depend on the size of the local larval habitats and the prevailing prices for labor, fuel, equipment and the control agent.

The usefulness of GIS and digital images in the control of zoonotic diseases is not necessarily

limited to assessment of larval habitats. The size of urban areas suitable for ultra-low volume (ULV) sprays could be assessed remotely. The technology could be used to detect and assess the size of ecotones important in the epidemiology of such diseases as hantavirus pulmonary syndrome (*Sin nombre* virus disease), scrub typhus, and Lyme disease. Once the size of the area is known, the cost of treatment can be calculated and compared to that of other disease control methods.

In this study, remote sensing and GIS were used to investigate the relationship between the size of the larval habitats and the at-risk population with regard to the cost of malaria control through chemoprophylaxis or larviciding. Neither method was uniformly cheaper; cost varied as a function of population and habitat size. This conclusion is intuitive, but the techniques described in this dissertation provide a method of quantifying the relationship.

Of course, numerous other variables impact on the decision-making process in malaria control. One of the most important variables that is not addressed in this study is the relative effectiveness of the

various methods. In the case of malaria control in the ROK, the efficacy of chemoprophylaxis taken as indicated is considered to be very high. This efficacy is reduced significantly by non-compliance, however, and the degree to which non-compliance and drug-resistance contribute to chemoprophylactic failures is difficult to ascertain. Conversely, the efficacy of larvicidal applications for malaria control is not defined at all in the ROK. Many attempts to fight malaria through larval control have failed, especially when the primary vector is very efficient (anthropophagic and endophilic). Theorectically, an efficient vector could transmit malaria even though vector populations are very low. This may not be the case in the ROK, where the assumed primary vector is far from efficient. *Anopheles sinensis* is known to be zoophilic and is more likely to remain outdoors. Significant reductions in the populations of an inefficient vector, like *An. sinensis*, may be much more likely to achieve significant levels of disease control than would similar reduction of an efficient vector, like *An. gambiae*. Even small populations of the latter species have contributed to significant disease transmission.

Some larval control programs, such as the Rockefeller Foundation's program using Paris Green in Brazil, have been used to effectively eliminate this species, but the situation in the ROK is not comparable. In short, any decision to rely on larviciding for malaria control in the ROK would have to be preceded by a field trial to at least ascertain the effectiveness of the selected agent for reducing the vector populations. The next step would be to observe the effect of reduced vector populations on the transmission of disease.

Other variables that impact on the decision-making process include local customs or cultural barriers, available expertise, local weather conditions, politics, healthcare infrastructure and the availability of equipment, medication and supplies. In some places in the Orient, the use of *Bacillus* formulations is discouraged due to the perceived impact of these agents on the silkworm industry (personal observation). This is just one example of how local perceptions can limit disease control options, even on U.S. bases in foreign countries. Similarly, the political situation in the ROK limits the use of aerial spray for the application

of pesticides, especially near the DMZ. All of these factors play some role in the decision as to which disease control methods to use; cost is just one of many.

In this study, the estimated costs of two disease-control programs are compared. Another method of comparison would be a cost-effectiveness study. For example, one could estimate the cost per malaria case prevented, or the cost per sick-year prevented by a given disease control method. This would require the collection and analysis of epidemiologic data that is not currently available. The long incubation period observed in this variety of *Plasmodium* complicates the reporting of the disease, especially given that many cases are diagnosed back in the United States. These cases may not be consistently reported and may be difficult to separate from cases of malaria contracted elsewhere, as troops often spend short periods of time in a variety of malarious areas. This situation is complicated further by the large number of troop rotations among U.S. Army personnel in the ROK, making the capture of numerator and denominator data problematic. Disease reporting procedures are not always followed as public health billets may be

empty for long periods of time. During the field phase of this study, the Division Preventive Medicine Officer position for the 2nd Infantry Division was vacant for three months. U.S. military personnel rarely stay for more than one year in the ROK, so year-to-year consistency in disease reporting is difficult to maintain. All of these factors increase the difficulty of performing a reliable cost-effectiveness analysis. On the other hand, a direct cost-comparison like that described in this study, can be relatively straightforward and it answers the question most high-level military officials will want answered; it provides information on how much a control method will cost to initiate and maintain.

Study limitations

The most evident limitation of this study is the availability of only one year's data. With additional data, the relationship between vector larval abundance and various environmental parameters might become evident. Although it is not possible to continue the study at the present time, I would recommend that future researchers consider the cycle

of flooding and drying as a possible determinant of larval occurrence/abundance. The use of agricultural compounds and their effects on water quality, particularly nitrogen content, also warrant additional research. In addition, this study did not report any relationship between larval abundance and the presence of terrestrial vegetation along the perimeter of the larval habitats. Although most of the rice paddy perimeters were mowed periodically, some were not. Some ditches were also overgrown late in the season. Further work on plant identification and relationships with larval abundance would be desirable.

Due to the objective of the study, sampling was performed on a macroscale suitable for remote sensing. Larval sampling at a smaller scale, for instance microhabitats within rice paddy, would be more likely to identify relationships between environmental parameters and larval occurrence/abundance. Such studies would be the next logical step in the field biology of these malaria vectors.

There are several limitations with the taxonomic aspects of this study. At this writing, the gene sequences for the single specimen with both adult

218

and pupal characters of *An. lesteri* are not known.
Similarly, the sequences for the colonized *An.
sinensis* have not been completed. These sequences
will be determined in future research. In addition,
known *An. lesteri* have not been obtained for
comparison. In fact, Dr. Bruce Harrison, an imminent
mosquito taxonomist with experience in Oriental
culicids, questions the validity of the identification
of the Shanghai *An. sinensis* used as a standard
comparison in this study. To complete this study,
known *An. lesteri*, Cantonese *An. sinensis*, and more
Korean *An. lesteri* should be sequenced and compared
with RAPDs. A comparison with *An. anthropophagous*
would also be beneficial. At present, the colonized
An. sinensis and the putative *An. lesteri* from this
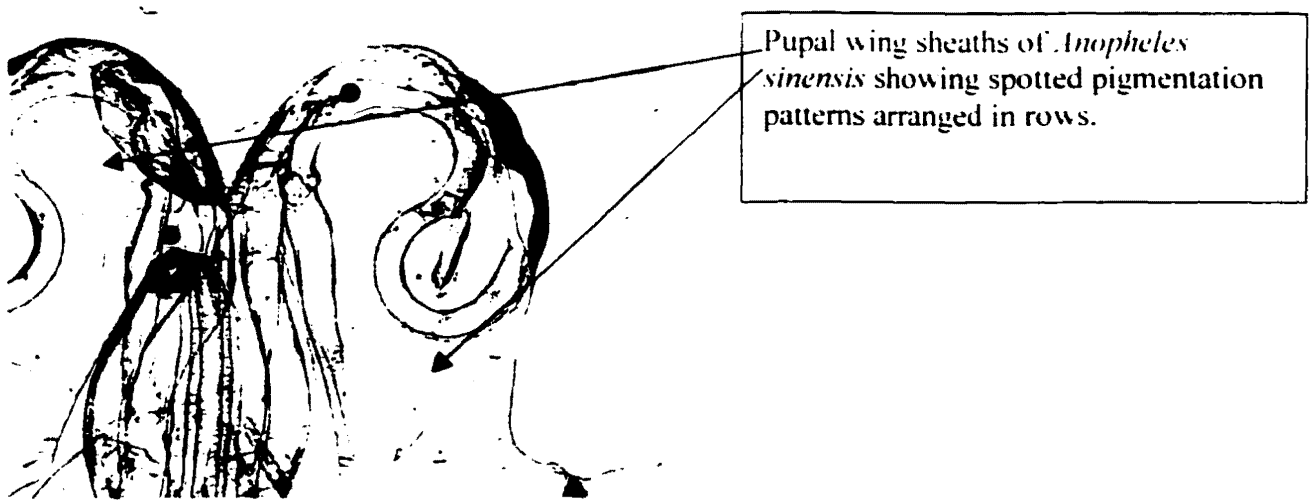study are being sequenced for the DII and COII genes.

The remote sensing aspects of this study could
be improved by including more U.S. Army camps as study
areas. This would not only add statistical validity
to the study, it would also allow larval surveillance
in other habitat types that may have been missed (i.e.
river, mountain and forest). This is particularly
important given the nearly complete absence of *An.
yatsushiroensis* in this collection. Given the

possible vector status of this species and the large number supposedly collected in adult studies, detection of the larval habitat of *An. yatsushiroensis* is essential.

Finally, this study would benefit from transect surveillance through the rice paddy environment. Although this study suggests that *An. sinensis* is clustered near the edge of the fields, this does not prove that other species do not have different distributions. With more time and access to translators, permission to enter the paddies might be obtained, allowing a more complete surveillance of larval distribution. The U.S. Army, in particular the 18th Medical Command, has expressed an interest in a continued relationship with USUHS. Perhaps future graduate students could refine aspects of this study in a long-term, multi-year program to investigate malaria vector biology and control in the ROK.

APPENDIX A

Pupal morphological characters for distinguishing

Anopheles sinensis and **An.** *lesteri*.

Pupal wing sheaths of *Anopheles sinensis* showing spotted pigmentation patterns arranged in rows.

Pupal wing sheaths of *Anopheles lesteri* showing cross-hatched pigmentation and spotted patterns
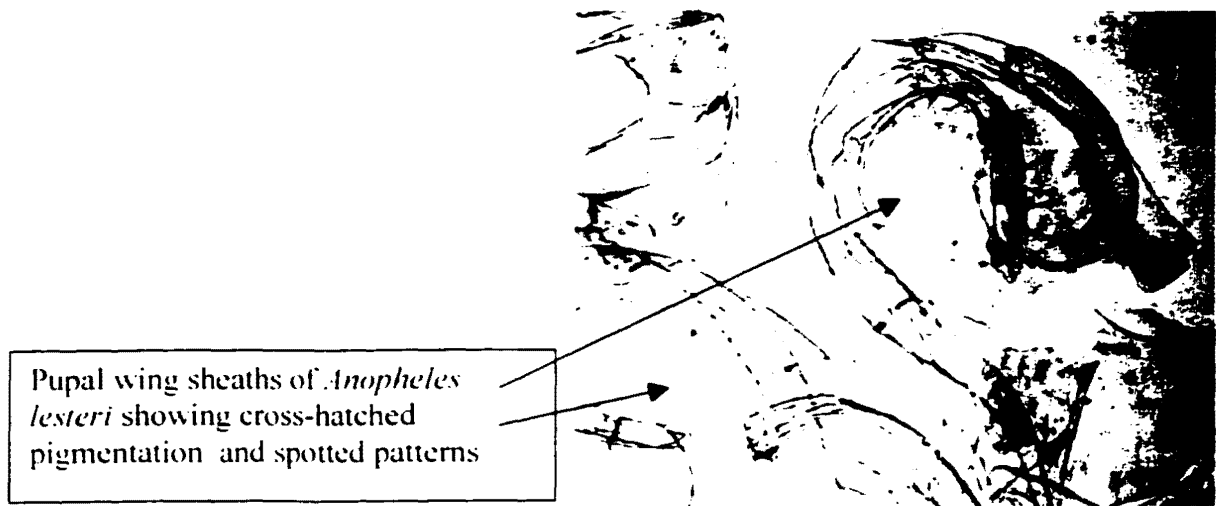
APPENDIX B

Partial sequences of two genes from South Korean

Anopheles

PARTIAL SEQUENCE OF COII GENE FROM 10 SOUTH KOREAN MOSQUITOES PUTATIVELY IDENTIFIED AS *AN. LESTERI* AND *AN. SINENSIS*. (Mutations noted in bold type)

lesteri 1 GAA CAT TGA CCA AAA AAT AAT CCA GGT CGA TTG ATT AAA AAA TTA ATT

lesteri 2 GAA CAT TGA CCA AAA AAT AAT CCA GGT CGA TTG ATT AAA AAA TTA ATT

lesteri 3 GAA CAT TGA CCA AAA AAT AAT CCA GGT CGA TTG ATT AAA AAA TTA ATT

lesteri 4 GAA CAT TGA CCA AAA AAT AAT CCA GGT CGA TTG ATT AAA AAA TTA ATT

lesteri 5 GAA CAT TGA CCA AAA AAT AAT CCA GGT CGA TTG ATT AAA AAA TTA ATT

sinensis 1 GAA CAT TGA CCA AAA AAT AAT CCA GGT CGA TTG ATT AAA AAA TTA ATT

sinensis 2 GAA CAT TGA CCA AAA AAT AAT CCA GGT CGA TTG ATT AAA AAA TTA ATT

sinensis 3 GAA CAT TGA CCA AAA AAT AAT CCA GGT CGA TTG ATT AAA AAA TTA ATT

sinensis 4 GAA CAT TGA CCA AAA AAT AAT CCA GGT CGA TTG ATT AAA AAA TTA ATT

sinensis 5 GAA CAT TGA CCA AAA AAT AAT CCA GGT CGA TTG ATT AAA AAA TTA ATT

lesteri 1 TGA TTT AAT CGT CCT GGT GTA GCA TCT ACC TTT ACT CCT AAA GAT GGT

lesteri 2 TGA TTT AAT CGT CCT GGT GTA GCA TCT ACC TTT ACT CCT AAA GAT GGT

lesteri 3 TGA TTT AAT CGT CCT GGT GTA GCA TCT ACC TTT ACT CCT AAA GAT GGT

lesteri 4 TGA TTT AAT CGT CCT GGT GTA GCA TCT ACC TTT ACT CCT AAA GAT GGT

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lesteri 5   TGA TTT AAT CGT CCT GGT GTA GCA TCT ACC TTT ACT CCT AAA GAT GGT

sinensis 1  TGA TTT AAT CGT CCT GGT GTA GCA TCT ACC TTT ACT CCT AAA GAT GGT

sinensis 2  TGA TTT AAT CGT CCT GGT GTA GCA TCT ACC TTT ACT CCT AAA GAT GGT

sinensis 3  TGA TTT AAT CGT CCT GGT GTA GCA TCT ACC TTT ACT CCT AAA GAT GGT

sinensis 4  TGA TTT AAT CGT CCT GGT GTA GCA TCT ACC TTT ACT CCT AAA GAT GGT

sinensis 5  TGA TTT AAT CGT CCT GGT GTA GCA TCT ACC TTT ACT CCT AAA GAT GGT


lesteri 1   ACT GTT CAT GAA TGT AAT ACA TCA GTA GCT GTA ACT AAA ATT CGA ATT

lesteri 2   ACT GTT CAT GAA TGT AAT ACA TCA GTA GCT GTA ACT AAA ATT CGA ATT

lesteri 3   ACT GTT CAT GAA TGT AAT ACA TCA GTA GCT GTA ACT AAA ATT CGA ATT

lesteri 4   ACT GTT CAT GAA TGT AAT ACA TCA GTA GCT GTA ACT AAA ATT CGA ATT

lesteri 5   ACT GTT CAT GAA TGT AAT ACA TCA GTA GCT GTA ACT AAA ATT CGA ATT

sinensis 1  ACT GTT CAT GAA TGT AAT ACA TCA GTA GCT GTA ACT AAA ATT CGA ATT

sinensis 2  ACT GTT CAT GAA TGT AAT ACA TCA GTA GCT GTA ACT AAA ATT CGA ATT

sinensis 3  ACT GTT CAT GAA TGT AAT ACA TCA GTA GCT GTA ACT AAA ATT CGA ATT

sinensis 4  ACT GTT CAT GAA TGT AAT ACA TCA GTA GCT GTA ACT AAA ATT CGA ATT

sinensis 5  ACT GTT CAT GAA TGT AAT ACA TCA GTA GCT GTA ACT AAA ATT CGA ATT
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lesteri 1    TGA TTA TTT ATA GGT AAA ACA ATT CGA TTA TCA ACA TCT AAA AGT CGA

lesteri 2    TGA TTA TTT ATA GGT AAA ACA ATT CGA TTA TCA ACA TCT AAA AGT CGA

lesteri 3    TGA TTA TTT ATA GGT AAA ACA ATT CGA TTA TCA ACA TCT AAA AGT CGA

lesteri 4    TGA TTA TTT ATA GGT AAA ACA ATT CGA TTA TCA ACA TCT AAA AGT CGA

lesteri 5    TGA TTA TTT ATA GGT AAA ACA ATT CGA TTA TCA ACA TCT AAA AGT CGG

sinensis 1   TGA TTA TTT ATA GGT AAA ACA ATT CGA TTA TCA ACA TCT AAA AGT CGG

sinensis 2   TGA TTA TTT ATA GGT AAA ACA ATT CGA TTA TCA ACA TCT AAA AGT CGA

sinensis 3   TGA TTA TTT ATA GGT AAA ACA ATT CGA TTA TCA ACA TCT AAA AGT CGG

sinensis 4   TGA TTA TTT ATA GGT AAA ACA ATT CGA TTA TCA ACA TCT AAA AGT CGA

sinensis 5   TGA TTA TTT ATA GGT AAA ACA ATT CGA TTA TCA ACA TCT AAA AGT CGA


lesteri 1    AAT CCA TTT GTT TCT AGT TCA TTT GTA GGA ATT ATA TAT GAA TCA AAT

lesteri 2    AAT CCA TTT GTT TCT AGT TCA TTT GTA GGA ATT ATA TAT GAA TCA AAT

lesteri 3    AAT CCA TTT GTT TCT AGT TCA TTT GTA GGA ATT ATA TAT GAA TCA AAT

lesteri 4    AAT CCA TTT GTT TCT AGT TCA TTT GTA GGA ATT ATA TAT GAA TCA AAT
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226

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lesteri 5    AAT CCA TTT GTT TCT AGT TCG TTT GTA GGA ATT ATA TAT GAA TCA AAT

sinensis 1   AAT CCA TTT GTT TCT AGT TCG TTT GTA GGA ATT ATA TAT GAA TCA AAT

sinensis 2   AAT CCA TTT GTT TCT AGT TCA TTT GTA GGA ATT ATA TAT GAA TCA AAT

sinensis 3   AAT CCA TTT GTT TCT AGT TCG TTT GTA GGA ATT ATA TAT GAA TCA AAT

sinensis 4   AAT CCA TTT GTT TCT AGT TCA TTT GTA GGA ATT ATA TAT GAA TCA AAT

sinensis 5   AAT CCA TTT GTT TCT AGT TCA TTT GTA GGA ATT ATA TAT GAA TCA AAT




lesteri 1    TCT AAA TTT AAA AAA TCA GAA TAT TCA TAG CTT CAA TAT CAT TGA TGA

lesteri 2    TCT AAA TTT AGA AAA TCA GAA TAT TCA TAG CTT CAA TAT CAT TGA TGA

lesteri 3    TCT AAA TTT AAA AAA TCA GAA TAT TCA TAG CTT CAA TAT CAT TGA TGA

lesteri 4    TCT AAA TTT AAA AAA TCA GAA TAT TCA TAG CTT CAA TAT CAT TGA TGA

lesteri 5    TCT AAA TTT AAA AAA TCA GAA TAT TCA TAG CTT CAA TAT CAT TGA TGA

sinensis 1   TCT AAA TTT AAA AAA TCA GAA TAT TCA TAG CTT CAA TAT CAT TGA TGA

sinensis 2   TCT AAA TTT AGA AAA TCA GAA TAT TCA TAG CTT CAA TAT CAT TGA TGA

sinensis 3   TCT AAA TTT AAA AAA TCA GAA TAT TCA TAG CTT CAA TAT CAT TGA TGA

sinensis 4   TCT AAA TTT AAA AAA TCA GAA TAT TCA TAG CTT CAA TAT CAT TGA TGA
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sinensis 5    TCT AAA TTT AAA AAA TCA GAA TAT TCA TAG CTT CAA TAT CAT TGA TGA



lesteri 1     CCA ACC GAC TTT AAT GTA ATT GAA GGT GTA TTA ATT TCG TCT ATT AAA

lesteri 2     CCA ACC GAC TTT AAT GTA ATT GAA GGT GTA TTA ATT TCG TCT ATT AAA

lesteri 3     CCA ACC GAC TTT AAT GTA ATT GAA GGT GTA TTA ATT TCG TCT ATT AAA

lesteri 4     CCA ACC GAC TTT AAT GTA ATT GAA GGT GTA TTA ATT TCG TCT ATT AAA

lesteri 5     CCA ACT GAC TTT AAT GTA ATT GAA GGT GTG TTA ATT TCG TCT ATT AAA

sinensis 1    CCA ACT GAC TTT AAT GTA ATT GAA GGT GTG TTA ATT TCG TCT ATT AAA

sinensis 2    CCA ACC GAC TTT AAT GTA ATT GAA GGT GTA TTA ATT TCG TCT ATT AAA

sinensis 3    CCA ACT GAC TTT AAT GTA ATT GAA GGT GTG TTA ATT TCG TCT ATT AAA

sinensis 4    CCA ACC GAC TTT AAT GTA ATT GAA GGT GTA TTA ATT TCG TCT ATT AAA

sinensis 5    CCA ACC GAC TTT AAT GTA ATT GAA GGT GTA TTA ATT TCG TCT ATT AAA



lesteri 1     TAT AAT AAA CGT AAA GAA GGA AAT GCA ATG AAT ATT AAA ATA ATT GCA

lesteri 2     TAT AAT AAA CGT AAA GAA GGA AAT GCA ATG AAT ATT AAA ATA ATT GCA

lesteri 3     TAT AAT AAA CGT AAA GAA GGA AAT GCA ATG AAT ATT AAA ATA ATT GCA
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lesteri 4    TAT AAT AAA CGT AAA GAA GGA AAT GCA ATG AAT ATT AAA ATA ATT GCA

lesteri 5    TAT AAT AAA CGT AAA GAA GGA AAT GCA ATG AAT ATT AAA ATA ATT GCA

sinensis 1   TAT AAT AAA CGT AAA GAA GGA AAT GCA ATG AAT ATT AAA ATA ATT GCA

sinensis 2   TAT AAT AAA CGT AAA GAA GGA AAT GCA ATG AAT ATT AAA ATA ATT GCA

sinensis 3   TAT AAT AAA CGT AAA GAA GGA AAT GCA ATG AAT ATT AAA ATA ATT GCA

sinensis 4   TAT AAT AAA CGT AAA GAA GGA AAT GCA ATG AAT ATT AAA ATA ATT GCA

sinensis 5   TAT AAT AAA CGT AAA GAA GGA AAT GCA ATG AAT ATT AAA ATA ATT GCA




lesteri 1    GGT AAA ACA GTT CAA ATA ATT TCA ATT GTT TGT CCA TGT AAT AAA TAA

lesteri 2    GGT AAA ACA GTT CAA ATA ATT TCA ATT GTT TGT CCA TGT AAT AAA TAA

lesteri 3    GGT AAA ACA GTT CAA ATA ATT TCA ATT GTT TGT CCA TGT AAT AAA TAA

lesteri 4    GGT AAA ACA GTT CAA ATA ATT TCA ATT GTT TGT CCA TGT AAT AAA TAA

lesteri 5    GGT AAA ACA GTT CAA ATA ATT TCA ATT GTT TGT CCA TGT AAT AAA TAA

sinensis 1   GGT AAA ACA GTT CAA ATA ATT TCA ATT GTT TGT CCA TGT AAT AAA TAA

sinensis 2   GGT AAA ACA GTT CAA ATA ATT TCA ATT GTT TGT CCA TGT AAT AAA TAA
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sinensis 3   GGT AAA ACA GTT CAA ATA ATT TCA ATT GTT TGT CCA TGT AAT AAA TAA

sinensis 4   GGT AAA ACA GTT CAA ATA ATT TCA ATT GTT TGT CCA TGT AAT AAA TAA

sinensis 5   GGT AAA ACA GTT CAA ATA ATT TCA ATT GTT TGT CCA TGT AAT AAA TAA


lesteri 1    CGA TTA GTG AAT TGA TTA AAT ATT AGT ATT CCT ATA ATA TAT CCA ACT

lesteri 2    CGA TTA GTG AAT TGA TTA AAT ATT AGT ATT CCT ATA ATA TAT CCA ACT

lesteri 3    CGA TTA GTG AAT TGA TTA AAT ATT AGT ATT CCT ATA ATA TAT CCA ACT

lesteri 4    CGA TTA GTG AAT TGA TTA AAT ATT AGT ATT CCT ATA ATA TAT CCA ACT

lesteri 5    CGA TTA GTA AAT TGA TTA AAT ATT AGT ATT CCT ATA ATA TAC CCA ACT

sinensis 1   CGA TTA GTA AAT TGA TTA AAT ATT AGT ATT CCT ATA ATA TAC CCA ACT

sinensis 2   CGA TTA GTG AAT TGA TTA AAT ATT AGT ATT CCT ATA ATA TAT CCA ACT

sinensis 3   CGA TTA GTA AAT TGA TTA AAT ATT AGT ATT CCT ATA ATA TAC CCA ACT

sinensis 4   CGA TTA GTG AAT TGA TTA AAT ATT AGT ATT CCT ATA ATA TAT CCA ACT

sinensis 5   CGA TTA GTG AAT TGA TTA AAT ATT AGT ATT CCT ATA ATA TAT CCA ACT
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230

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lesteri 1    AAA ATT GTA ATT ATT GTT AAA ATA AGA AGA GTA TGA TCA TGA AAA AAA

lesteri 2    AAA ATT GTA ATT ATT GTT AAA ATA AGA AGA GTA TGA TCA TGA AAA AAA

lesteri 3    AAA ATT GTA ATT ATT GTT AAA ATA AGA AGA GTA TGA TCA TGA AAA AAA

lesteri 4    AAA ATT GTA ATT ATT GTT AAA ATA AGA AGA GTA TGA TCA TGA AAA AAA

lesteri 5    AAA ATT GTA ATT ATT GTT AAA ATA AGA AGA GTG TGA TCA TGA AAA AAA

sinensis 1   AAA ATT GTA ATT ATT GTT AAA ATA AGA AGA GTG TGA TCA TGA AAA AAA

sinensis 2   AAA ATT GTA ATT ATT GTT AAA ATA AGA AGA GTA TGA TCA TGA AAA AAA

sinensis 3   AAA ATT GTA ATT ATT GTT AAA ATA AGA AGA GTG TGA TCA TGA AAA AAA

sinensis 4   AAA ATT GTA ATT ATT GTT AAA ATA AGA AGA GTA TGA TCA TGA AAA AAA

sinensis 5   AAA ATT GTA ATT ATT GTT AAA ATA AGA AGA GTA TGA TCA TGA AAA AAA



lesteri 1    TTT AAT TGT TCT ATT AAA GGA

lesteri 2    TTT AAT TGT TCT ATT AAA GGA

lesteri 3    TTT AAT TGT TCT ATT AAA GGA

lesteri 4    TTT AAT TGT TCT ATT AAA GGA

lesteri 5    TTT AAT TGT TCT ATT AAA GGA
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231

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sinensis 1   TTT AAT TGT TCT ATT AAA GGA

sinensis 2   TTT AAT TGT TCT ATT AAA GGA

sinensis 3   TTT AAT TGT TCT ATT AAA GGA

sinensis 4   TTT AAT TGT TCT ATT AAA GGA

sinensis 5   TTT AAT TGT TCT ATT AAA GGA
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PARTIAL SEQUENCE OF DII GENE FROM *ANOPHELES SINENSIS*

TCC CGT AGG TAC CTC GAT TAG TTA GTA CAT CGT AGA TCC GGA

GTC GTG CTC GGC TCA GGC TTT CGC CCT TGA ACA TGC CCA AAC

ATG CGC TTC ACA CTT CTC TAG TTC ACT TCA ATG CCC ATC ACG

CAT CCA ACG GCA CAC CGG AAC TAG CCG AAC TCG AGA CCG GAG

CCT CGA TCC CCC GTT CGC GAC GCC GGC CTT AAG CTG AGC GCC

ACT ACT AGA GGG TCG ACC GCA TGT TCT GGG ACC TGA TGT TGG

ATA TGC TCG CAG CAA CCG GAG TCG CCG CTC GCA TTT GGT TTG

CGT AAT GGA TCA CGA TGT CCG CAC TCG TTC CTG GCT AGG GTC

AAG GCA TAC AGA AGG CCC CCT TCT GCG TGA CAG CCA AAA CCC

ACG ATG CCA GGA ACT ACG GAT AAT CGA GTT CAA CAG GCT TTA

CAC CCT CGG CAG TTT CAC GTA CTA TTT GAC TCT CTA TTC AGA

GTG CTT TTC AAC TTT CCC ACA CGG TAC TTG TTT ACT ATC GGT

CTC ATG GTC GTA TTT AGC TTT AGA AGG AGT TTA CCT CCC ACT

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APPENDIX C

Negative images of PCR-RAPD gels used to investigate reliability of adult morphological characters in identification of Korean malaria vectors.
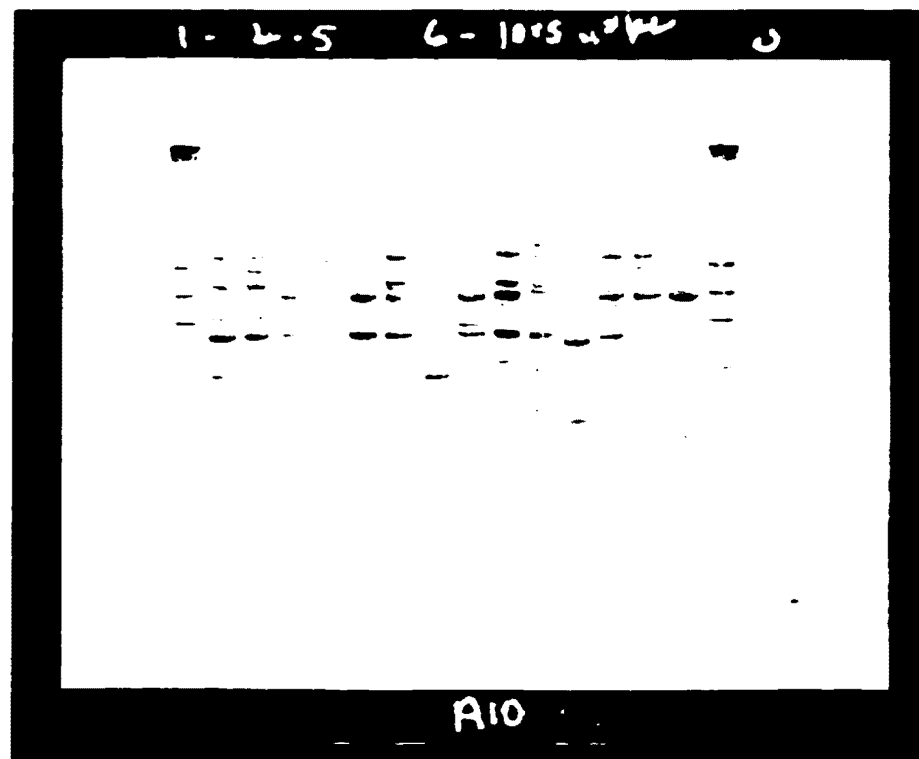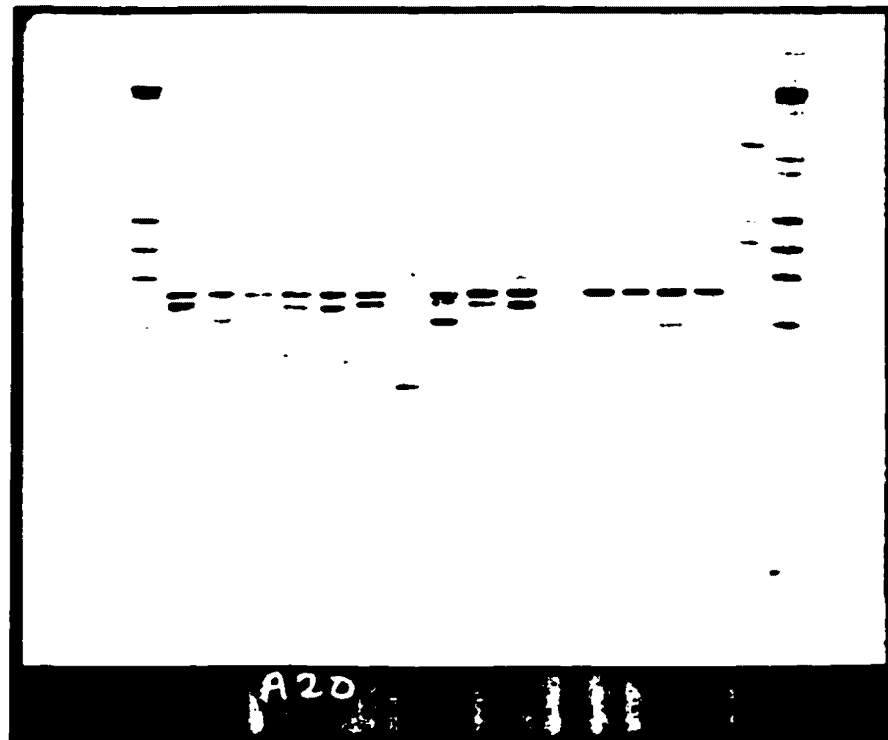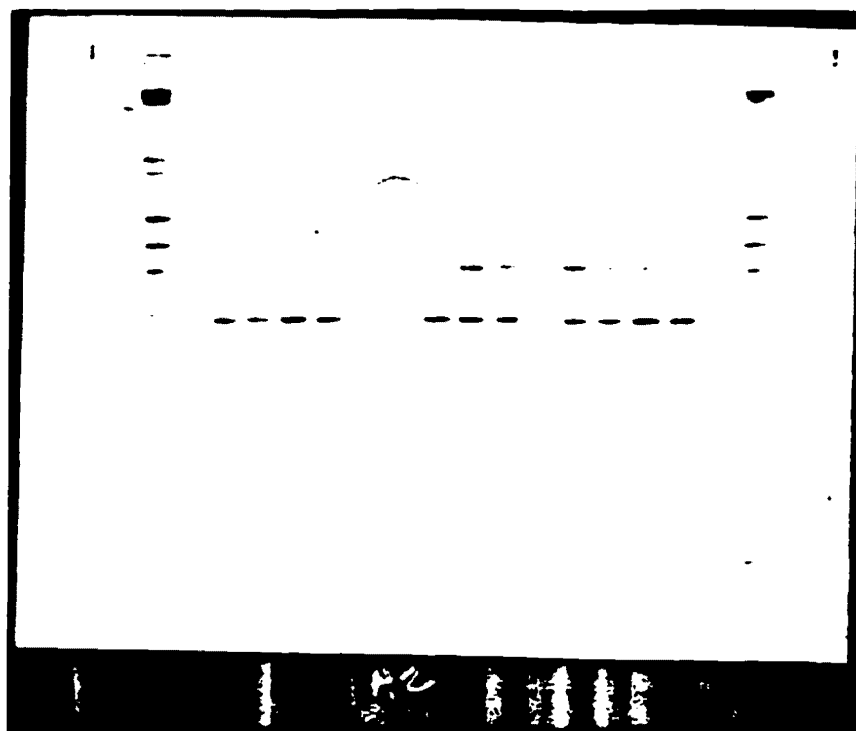
Figure 1. RAPD-PCR gel (negative image) of *Anopheles*
mitochondrial DNA (Primer A10). Molecular weight markers
(lanes 1 and 16), putative *An. lesteri* from South
Korea(lanes 2-6), *An. sinensis* from South Korea (lanes 7-
11), *An. lesteri* identified with pupal characteristics
(lane 12), colonized Chinese *An. sinensis* (lanes 13-15).
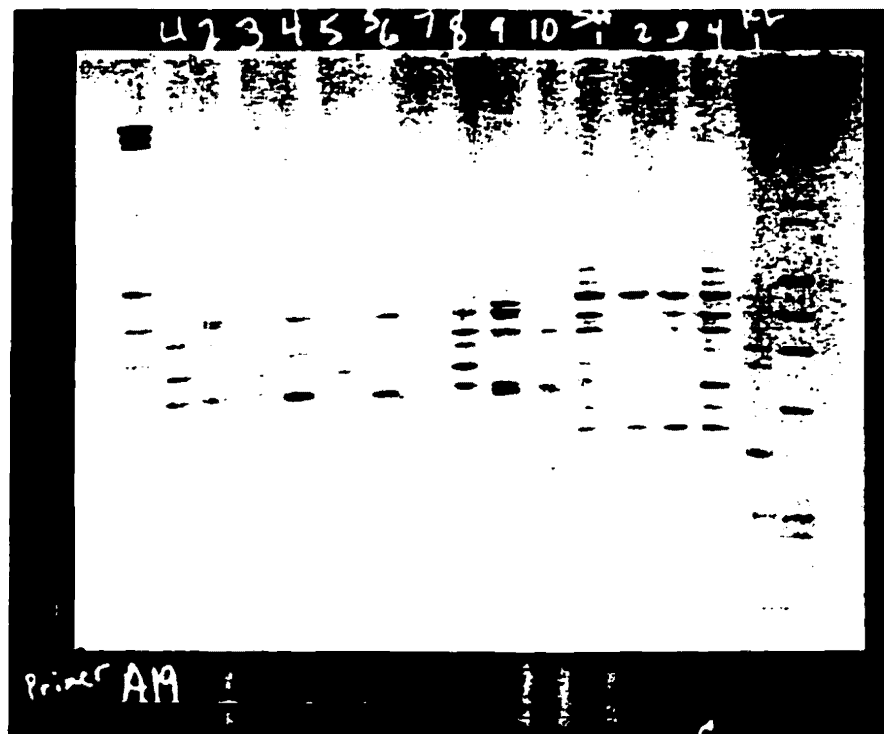
Figure 2. RAPD-PCR gel (negative image) of *Anopheles*

mitochondrial DNA (Primer A19). Molecular weight markers

(lanes 1 and 17), putative *An. lesteri* from South

Korea(lanes 2-6), *An. sinensis* from South Korea (lanes 7-

11), colonized Chinese *An. sinensis* (lanes 12-15), *An.*

lesteri identified with pupal characteristic (lane 16).

Figure 3. RAPD-PCR gel (negative image) of *Anopheles*
mitochondrial DNA (Primer A20). Molecular weight markers
(lanes 1 and 18), putative *An. lesteri* from South
Korea(lanes 2-6), *An. sinensis* from South Korea (lanes 7-
11), *An. lesteri* identified with pupal characteristics
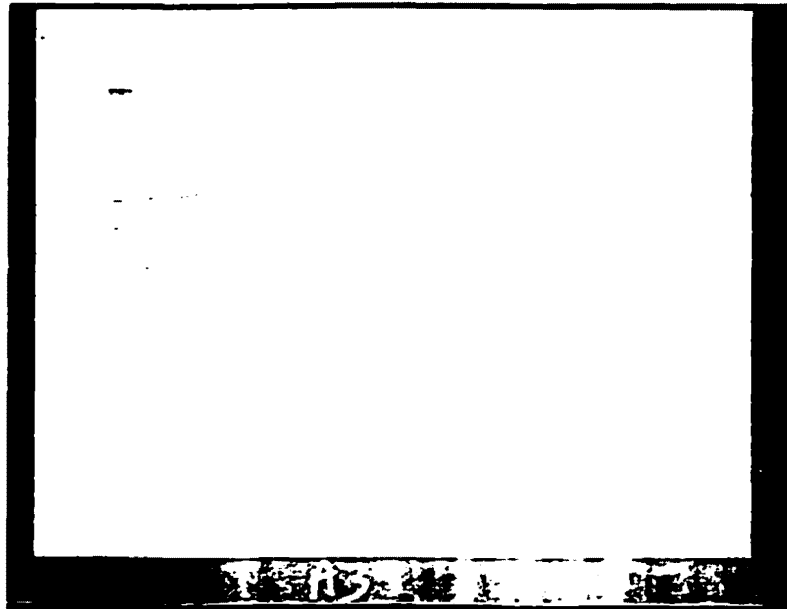(lane 12), colonized Chinese *An. sinensis* (lanes 13-16),
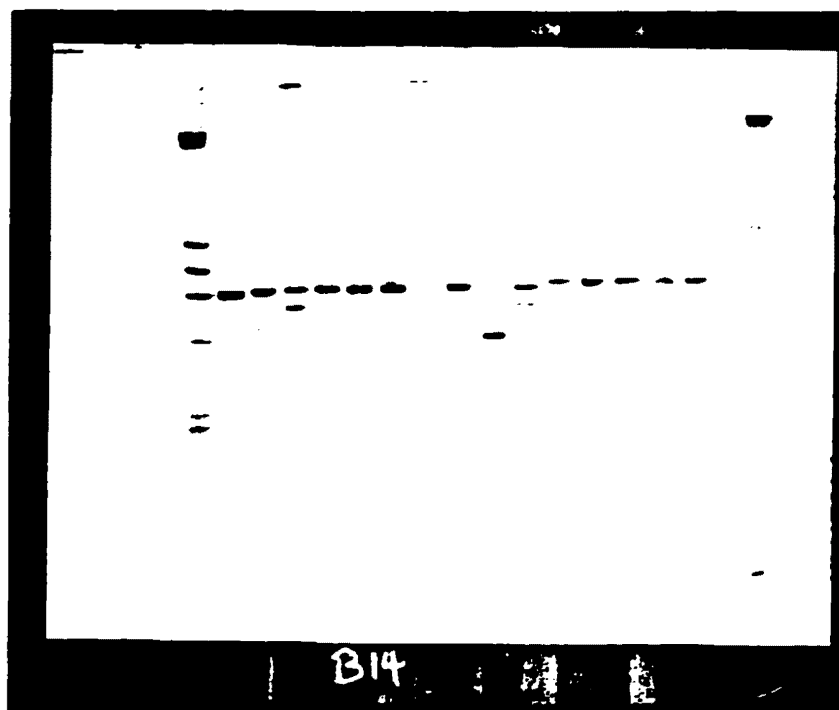control (lane 17).

237

Figure 4. RAPD-PCR gel (negative image) of *Anopheles*

mitochondrial DNA (Primer A2). Molecular weight markers

(lanes 1 and 18), putative *An. lesteri* from South

Korea(lanes 2-6), *An. sinensis* from South Korea (lanes 7-

11), *An. lesteri* identified with pupal characteristics

(lane 12), colonized Chinese *An. sinensis* (lanes 13-16),

control (lane 17).

Figure 5. RAPD-PCR gel (negative image) of *Anopheles*
mitochondrial DNA (Primer A3). Molecular weight markers
(lanes 1 and 17), putative *An. lesteri* from South
Korea(lanes 2-6), *An. sinensis* from South Korea (lanes 7-
11), *An. lesteri* identified with pupal characteristics
(lane 12), colonized Chinese *An. sinensis* (lanes 13-15),
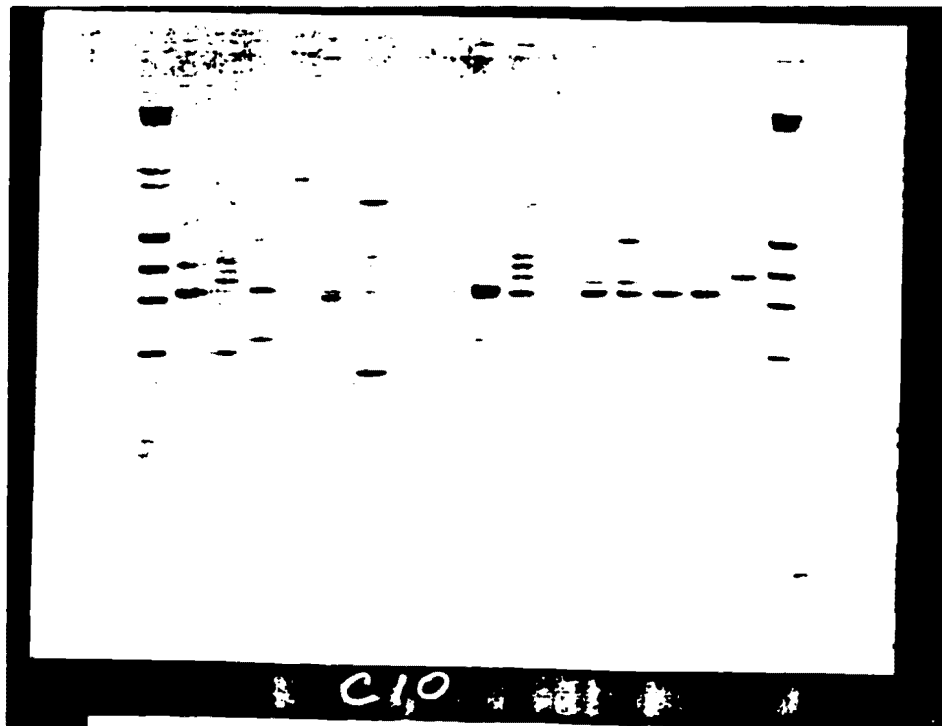control (lane 16).

Figure 6. RAPD-PCR gel (negative image) of *Anopheles* mitochondrial DNA (Primer B14). Molecular weight markers (lanes 1 and 18), putative *An. lesteri* from South Korea(lanes 2-6), *An. sinensis* from South Korea (lanes 7-11), *An. lesteri* identified with pupal characteristics (lane 12), colonized Chinese *An. sinensis* (lanes 13-16), control (lane 17).
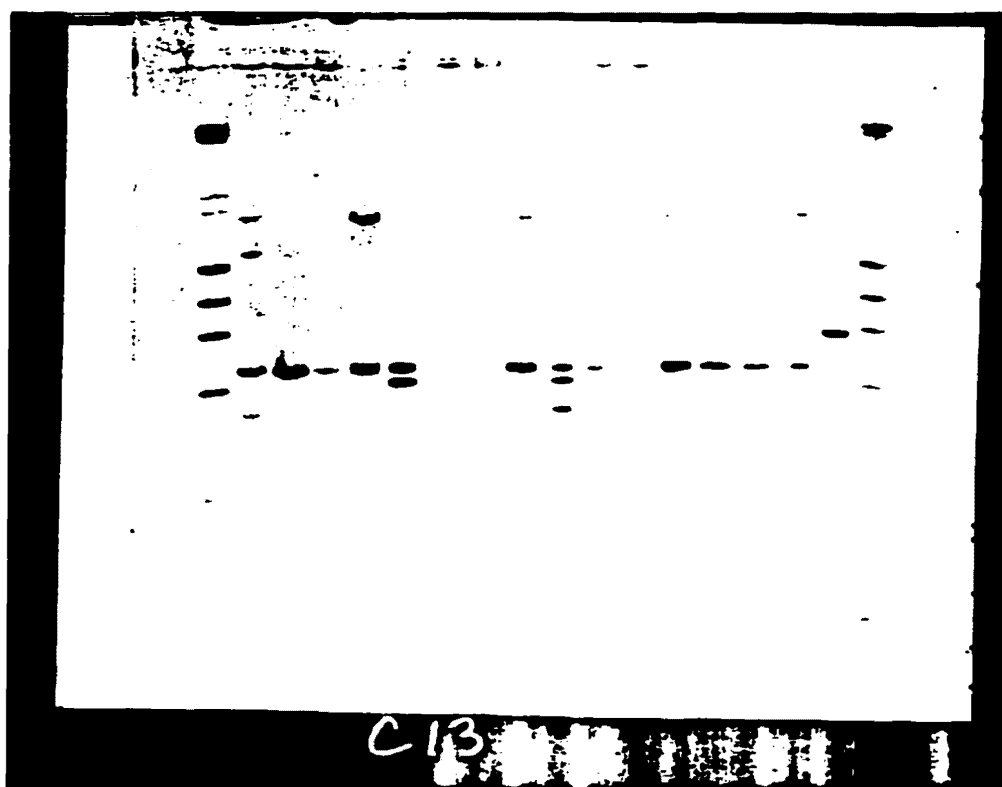
Figure 7. RAPD-PCR gel (negative image) of *Anopheles*
mitochondrial DNA (Primer C10). Molecular weight markers
(lanes 1 and 18), putative *An. lesteri* from South
Korea(lanes 2-6), *An. sinensis* from South Korea (lanes 7-
11), *An. lesteri* identified with pupal characteristics
(lane 12), colonized Chinese *An. sinensis* (lanes 13-16),
control (lane 17).

Figure 8. RAPD-PCR gel (negative image) of *Anopheles*
mitochondrial DNA (Primer c13). Molecular weight **markers**
(lanes 1 and 18), putative *An. lesteri* from South
Korea(lanes 2-6), *An. sinensis* from South Korea (lanes 9-
11), colonized Chinese *An. sinensis* (lanes 13-16), control
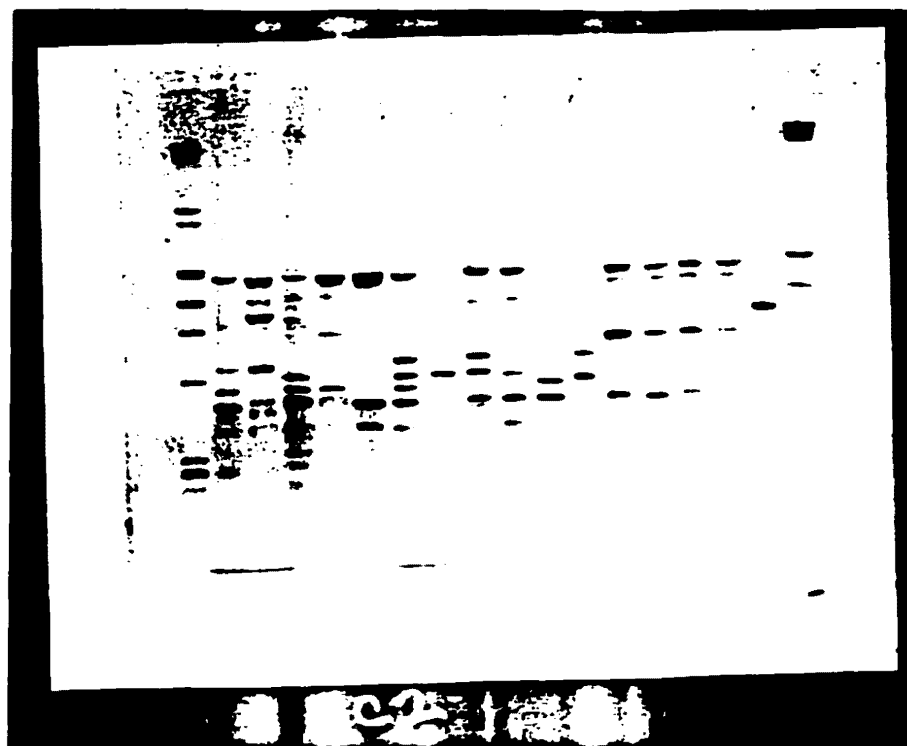(lane 17).

Figure 9. RAPD-PCR gel (negative image) of *Anopheles* mitochondrial DNA (Primer C2). Molecular weight markers (lanes 1 and 18), putative *An. lesteri* from South Korea(lanes 2-6), *An. sinensis* from South Korea (lanes 7-11), *An. lesteri* identified with pupal characteristics (lane 12), colonized Chinese *An. sinensis* (lanes 13-16), control (lane 17).
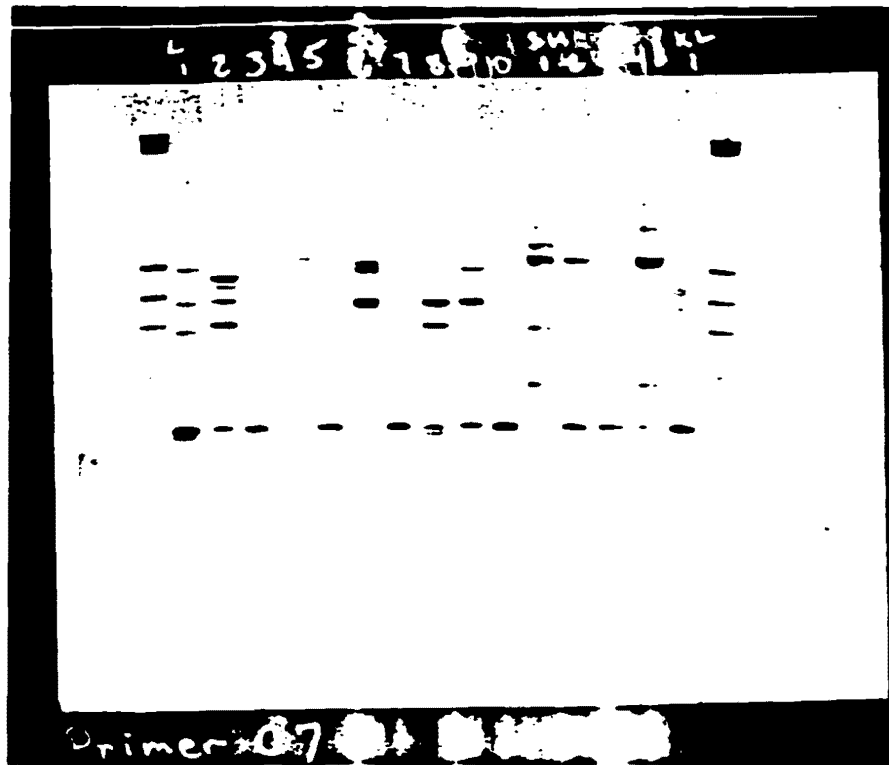
Figure 10. RAPD-PCR gel (negative image) of *Anopheles* mitochondrial DNA (Primer C7). Molecular weight markers (lanes 1 and 17), putative *An. lesteri* from South Korea (lanes 2-6), *An. sinensis* from South Korea (lanes 7-11), colonized Chinese *An. sinensis* (lanes 12-15), *An. lesteri* identified with pupal characteristics (lane 16).

Figure 11. RAPD-PCR gel (negative image) of
Anopheles mitochondrial DNA (Primer C20). Molecular
weight markers (lanes 1 and 18), putative *An. lesteri*
from South Korea (lanes 2-6), *An. sinensis* from South
Korea (lanes 7-11), *An. lesteri* identified with
pupal characteristics (lane 12), colonized Chinese
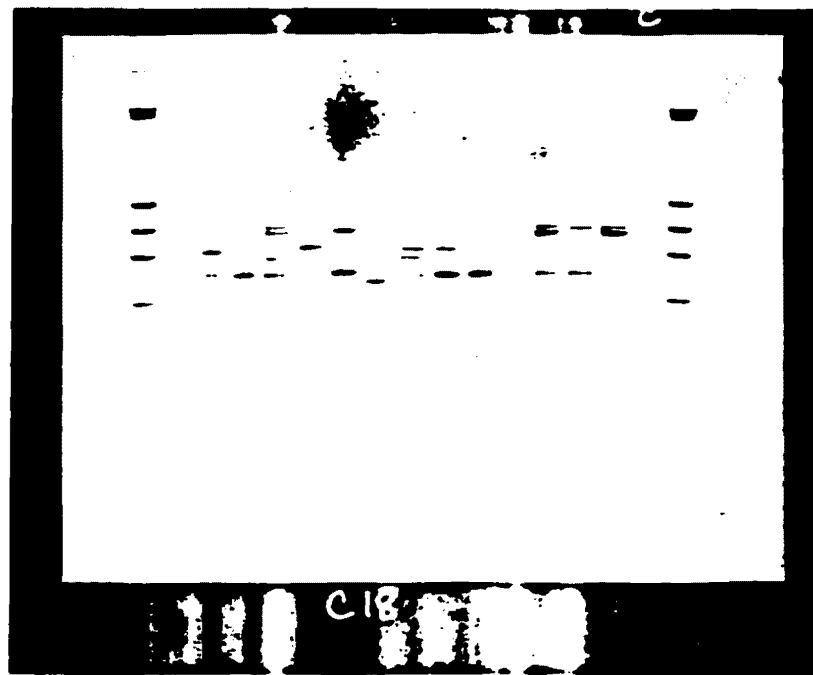*An. sinensis* (lanes 13-16), negative control (lane
17).

Figure 12. RAPD-PCR gel (negative image) of
Anopheles mitochondrial DNA (Primer C18). Molecular
weight markers (lanes 1 and 17), putative *An. lesteri*
from South Korea (lanes 2-6), *An. sinensis* from South
Korea (lanes 7-11), *An. lesteri* identified with pupal
characteristics (lane 12), colonized Chinese *An.
sinensis* (lanes 13-15), negative control (lane 16).